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Bacteriological

Reviews

A Publication of the American Society for Microbiology

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that they continually
open new fields to
our vision—PASTEUR*

Second International Conference
on Aerobiology
(Airborne Infection)

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BACTERIOLOGICAL REVIEWS

AIMS AND SCOPE

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Second International Conference on Aerobiology (Airborne Infection)

Chicago, Illinois
29–31 March 1966

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Foreword

The meeting termed the First International Conference on Aerobiology was held in October 1963 on the campus of the University of California at Berkeley and was cosponsored by the Naval Biological Laboratory and the University of California. The proceedings were published by the Naval Biological Laboratory as *A Symposium on Aerobiology, 1963*. Prior to this conference, however, the "key" conference which actually originated the series was held in December 1960 at Miami Beach, sponsored by the National Research Council with support from the National Institutes of Health and the U.S. Army Biological Laboratories. The proceedings were published as the September 1961 issue of *Bacteriological Reviews*. The present Second International Conference on Aerobiology continues the series of reports and discussions related to recent investigations in aerobiological fields.

Since the Berkeley conference, research in airborne infection has been stimulated by the problems arising from the increasing concentrations of military recruits in the various training centers. This, in turn, has caused an increase in research in aerobiology in general in order to provide information relating to the basic parameters of biological aerosols and to develop or improve the technology for such investigations. This interest will certainly continue, and conferences of this type can be anticipated at roughly 3-year intervals.

The Program Committee is indebted to the Session Chairmen for their time and competence and to many individuals from the U.S. Army Biological Laboratories and the Illinois Institute of Technology Research Institute for their willing efforts in arranging and conducting the conference.

ELWOOD K. WOLFE
General Chairman

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BACTERIOLOGICAL REVIEWS

Contents continued

Airborne Infections—the Past and the Future[1]

HARRY F. DOWLING

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Ebb and flow is as characteristic of research as of other activities of man. The transmission of infections through the air is a good example. Interest in airborne infections has had three flood tides in the past four centuries. The first was a long one. We can trace its beginning to 1546, when Fracastorius published his theory of contagion and explained that transmission of infections might occur by simple contact, by indirect contact, or by transmission from a distance. From that time on, the concept of airborne disease captured men's minds. "This most excellent canopy, the air," says Hamlet, "why it appeareth nothing to me but a foul and pestilent congregation of vapors." Hence, in times of epidemics the air was filled with the smoke of sulfur, camphor, tar, or pitch, and people religiously shut out the night air, walked away from noisome smells, or, if they couldn't avoid them, held perfumed kerchiefs to their noses.

The idea that diseases were spread by bad air (i.e., malaria) or by the influence (i.e., influenza) of planets, carried through the air, was too simple and naive an explanation. It was eventually discredited because attempts to apply it failed to stop the spread of infections. By the middle of the 19th century, it had been almost completely discarded by the medical profession (1). Pasteur's demonstration in 1861 that bacteria were consistently present in the unsterilized air and Lister's report in 1867 of the control of suppuration of wounds by measures which included a phenol spray should have stimulated everyone to vigorous attempts to control airborne infections. But this did not happen—for several reasons. For one thing, as I have said, all of the measures that could be conceived for limiting the spread of infection by air had already been tried and had failed. Also, it became evident to those surgeons who studied Lister's techniques carefully (rather than opposing them violently or aping them completely, as most did), that his method owed its success mainly to the suppression of contact infections. This was verified when Macewen about 1880 successfully used instruments and dressings sterilized by boiling and discarded the phenol spray. Finally, other public health measures had been successful and here, as elsewhere, nothing succeeds like success. To understand this, let us try to picture sanitary

conditions in our cities around 1850. As Rosenberg (5) so aptly put it:

> "A standing joke maintained that city water was far better than any other, since it served as a purgative as well as for washing and cooking. Most people were sensible enough not to drink it, except when forced by poverty or betrayed through inadvertence."

And Coleridge exclaimed,

> "I counted two-and-seventy stenches
> All well defined, and several stinks
>
> . . . . .
>
> The river Rhine, it is well known,
> Doth wash your city of Cologne;
> But tell me, nymphs! what power divine
> Shall henceforth wash the river Rhine?"

Since filth and putrescence were associated with disease in people's minds, the campaign for clean water gathered momentum. Paralleling this was the concept that fevers resulted from noxious exhalations from putrid animal and vegetable matter. Hence, methods for disposing of waste and sewage were developed gradually during the last half of the 19th century. The results were apparent in the dramatic drop in the mortality and morbidity from water-borne and insect-borne diseases at the turn of the century.

The emphasis, therefore, was on spread by the fecal-oral route and by direct contact. Airborne infections were too hard to understand, and techniques for their control were too complex. So matters stood at the end of World War I. Soon thereafter, a second wave of interest in airborne infections developed.

In 1917, Stillman (6) reported that he had cultured types I and II pneumococci in dust collected from homes of patients with pneumonia caused by the same types. He was able to grow microorganisms from the lungs of mice after exposure to a mist containing pneumococci (7), and he also produced pneumonia with these mists after partially immunizing mice and depressing them with alcohol (8). Branch and Stillman (2) also reported that pneumonia was produced in mice by the inhalation of β-hemolytic streptococci and *Klebsiella*. These observations were soon overshadowed by other studies in animals of pneumonia

produced by the installation of bacteria, especially the experiments of Robertson and his associates (4) on dogs, which extended over a number of years and added greatly to our knowledge of the pathogenesis of, and immunity from, pneumococcal pneumonia. Valuable as these latter studies were, they diverted interest away from the production of pneumonia by inhalation, since intrabronchial instillation was simpler and surer. Furthermore, the control of pneumonia took a hopeful turn in another direction; good results were obtained from serum therapy, followed by the greater successes of sulfonamides and antibiotics. Thus, the second wave of interest in airborne infections petered out. But the third wave was already forming.

In 1934, Wells introduced the concept of droplet nuclei (9) and followed this with a series of theoretical and applied experiments which broke the logjam and released a new flood of investigations. These investigations were reinforced by the rising interest in the spread of streptococcal infections in army barracks and of staphylococcal infections in hospitals, in the dissemination of viral and fungal infections, and finally in the chemical pollution of the atmosphere. Today we are on the crest of this wave of interest. Let us look at where we are and where we are travelling. To start with the sources of airborne infections, many studies have been made with sick and well persons as sources, but we still are not always sure where the microorganisms originate in the person who disseminates them and how they become dispersed in the air. What is the relative role of the upper and lower respiratory tract and of the skin? Some people spread streptococci and staphylococci more readily than others. We do not aways know why.

Studies have been made on the environment as a source of infection. These have been especially productive in relation to fungi. On the other hand, the principles by which infections are spread in the dust of rooms are only partially comprehended.

Studies have been made on the transmission of infection and the role of droplet nuclei and other small particles in the spread of various diseases, and great progress has been made in this area. I should like to suggest that such studies should not neglect the effect of temperature and humidity on microorganisms while they are in the air. For instance, we still have no idea why the common cold is more prevalent in the colder months. From experiments which my associates and I reported (3), it appears that the explanation does not lie in the chilling of the recipient. Does the virus remain alive longer in cold weather, or in hot dry buildings? Or is crowding of people in the winter

months the sole explanation? Finally, with regard to the relationship of microorganisms and chemicals in the air, studies have been made on chemicals which will prevent the spread of bacteria in the air, and some promising results have been obtained with the glycols. On the other hand, we are at present far from knowing whether air pollution augments airborne infection, and, if so, how.

A great deal more attention can be directed toward the recipient. We have a good idea how far microorganisms go into the lung during inhalation and how this is affected by particle size. We are studying the role of the mucus sheath, the cilia, phagocytosis, and local immunity, but we have only scratched the surface so far. Techniques are at hand with which to dig a great deal deeper, and many of them will be reported on in this Conference. The isolation of many of the respiratory viruses and extensive experience with the production of viral infections in volunteers makes it possible, for instance, to study dissemination, spread, and inception of airborne infections in man, something that we could not safely do with bacteria or fungi. Also, the rapid multiplication of antibiotics enables us to try to stop bacteria from growing in air passages even after they have arrived in large numbers through the air.

Finally, there is the control of airborne infections. Because earlier attempts to prevent these infections by ultraviolet light and chemical disinfection were not uniformly successful, the atmosphere in a roomful of investigators of respiratory infections seems itself to be infected with pessimism. Need this be so? The technology of ventilation is advancing all the time, and increasing numbers of people are living the year around in artificial atmospheres which eagerly wait for someone to make them nearly germ-free. Besides, we have failed to capitalize on one strong force, the increasing chemical pollution of the air we breathe. I remember as a boy seeing a performer in a circus dive into a glass tank and eat a banana under water. I was horrified to think that he was eating food that was coated with the bacteria he carried with him every time he dived in. And yet, we are not doing anything different in breathing air contaminated with bacteria. Air in a crowded room may be as contaminated as water in the average farm-pond. But just as our ancestors paid no attention to the water so long as it looked clear, so the public pays no attention to the air when it seems pure. Now that the stench and the irritation from chemical wastes are becoming daily more obnoxious, can we not mobilize interest in cleansing the air? And just as ridding the water of foul tastes was the beginning of purifying it of bacteria, so we may succeed in doing both to the air.

What would happen to our immunity if we breathed in no microorganisms year in and year out? We know the answer from bitter experience with measles in isolated populations or with polio-myelitis in the more advanced countries. To keep up our protection we will have to vaccinate. But how can we immunize against the hundreds of bacteria and viruses, to which people now develop enough immunity to produce a tolerable equi-librium? Perhaps it can be done by isolating the purified antigens and combining them in feasible doses for injection. But it will take a long time to find these antigens, and it is by no means certain that we can prepare all the vaccine necessary or persuade the people to take them if we do. This is where immunization by inhalation comes in. The pioneer studies of vaccination against tetanus and tularemia have shown the way, but to my mind this subject has not received nearly the attention that is its due. The methods of inhalation have been perfected; the bacteria and most of the vi-ruses have been isolated; now the time is ripe to extend the few studies that have been made. One might even visualize, a few years from now, school children and office workers receiving their im-munizations without fuss or discomfort while they are sitting at their desks going about their regular work, or, alternatively, inhalo-mobiles that would park outside a school, an office building, or a shopping center, attracting people inside for their inhalations much as X rays for tuberculosis are taken nowadays.

What I have just said may sound grandiose, but I am using this picture deliberately to point up the main theme of my discussion. We have had earlier waves of interest in airborne diseases, but they have died out because other methods of control of infections were more successful and because adequate techniques were not at hand to study and control infections transmitted through the air. As a result, control of contact infections and of food-borne, insect-borne, and water-borne infections is far ahead of control of airborne in-

fection. But the present wave of interest is high, and the techniques are at hand. To paraphrase Brutus,

> "There is a tide in th' pursuit of
>   knowledge,
> Which, taken at the flood, will bring
>   success.
> On such a sea are we now afloat
> And we can take the measures at our
>   hand
> And win our ventures."

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Mechanisms of Antibacterial Action in the Respiratory System

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INTRODUCTION

Perhaps the most striking finding in relation to the antibacterial activity in the respiratory system is the sterility of the bronchopulmonary apparatus from the primary bronchi downward. That these structures are ordinarily sterile, despite the continuous entry of droplet nuclei containing bacteria, has been known for more than 50 years (3). The mechanism by which the bronchopulmonary tree retains its sterility under ordinary circumstances is just beginning to be understood.

Our attention was drawn to the problem by the considerations that if, in fact, the bronchopulmonary tree were normally sterile, and if in chronic bronchitis the lower bronchial secretions were ordinarily heavily populated with bacteria, there might be a basis for investigating the pathogenesis of chronic bronchopulmonary infections from the standpoint of breakdown of mechanism of local antibacterial activity. Initial investigations, therefore, were directed toward the study of the bacteriological flora of the bronchopulmonary secretions, with the use of extreme precautions to avoid contamination of the cultured material by bacteria coming from the upper respiratory passages. These studies (10) confirmed earlier observations (1, 11) that bacteria were rarely found in the bronchopulmonary secretions unless there was manifest inflammation or exudation. By the same token, when such exudation was found, the bacteria tended to be present in large numbers (>10⁴ colonies per milliliter of secretion), and often the bacteria in the bronchial secretions were

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not adequately represented in cultures of the sputum.

The present review is not intended as a detailed discussion of the implications of these findings in terms of bacteriological interpretation of cultures arising from the bronchopulmonary apparatus. Instead, attention will be directed toward the implication that the failure to find bacteria could be explained primarily on the basis of continued activity of a potent local antibacterial mechanism. That such a mechanism exists has been indicated by numerous studies in the past. For example, Stillman (16), working with pneumococci that had been instilled into the bronchi of mice, observed that the majority of these organisms were made nonviable by the lung shortly after instillation. Lurie, in his classic experiments on the fate of aerosolized tubercle bacilli, observed that as many as 100 organisms needed to be inhaled to set up one tubercle, even in genetically highly susceptible rabbits, and one or two orders of magnitude more bacteria were required to be inhaled to produce a tubercle in genetically more resistant strains of rabbit (12). It was apparent, then, that the overwhelming majority of the inhaled bacteria were being killed in some manner after inhalation.

BACTERIAL CLEARANCE IN THE NORMAL LUNG

To study the phenomenon of local killing in the lung, an aerosol apparatus was constructed out of simple and relatively inexpensive materials (8). The apparatus delivered more than 85% of its particles in the form of nuclei between 1 and 3 μ in diameter, and, in detailed studies of the function of the apparatus, it was found that a set of stand-

FIG. 1. *Disappearance of Staphylococcus aureus from the lungs of mice after administration by aerosol. The rapid disappearance and small standard deviations are especially noteworthy. (Reprinted from reference 8.)*
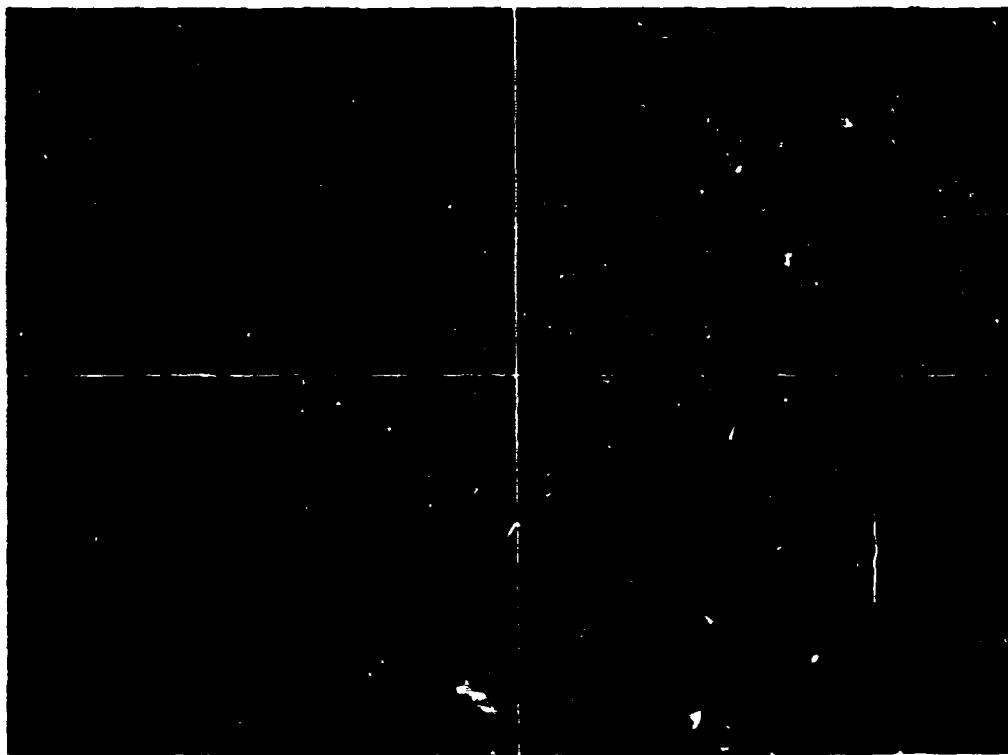
FIG. 2. *Lungs of mice taken 4 hr after exposure to aerosols containing Staphylococcus aureus. The sections have been stained with antibody to the Staphylococcus by use of fluorescein-labeled antibody, in accordance with methods given in reference 6. Note the intense staining of bacterial antigen in some cells lining the alveoli. In a few instances, discrete coccal bodies can also be seen.*
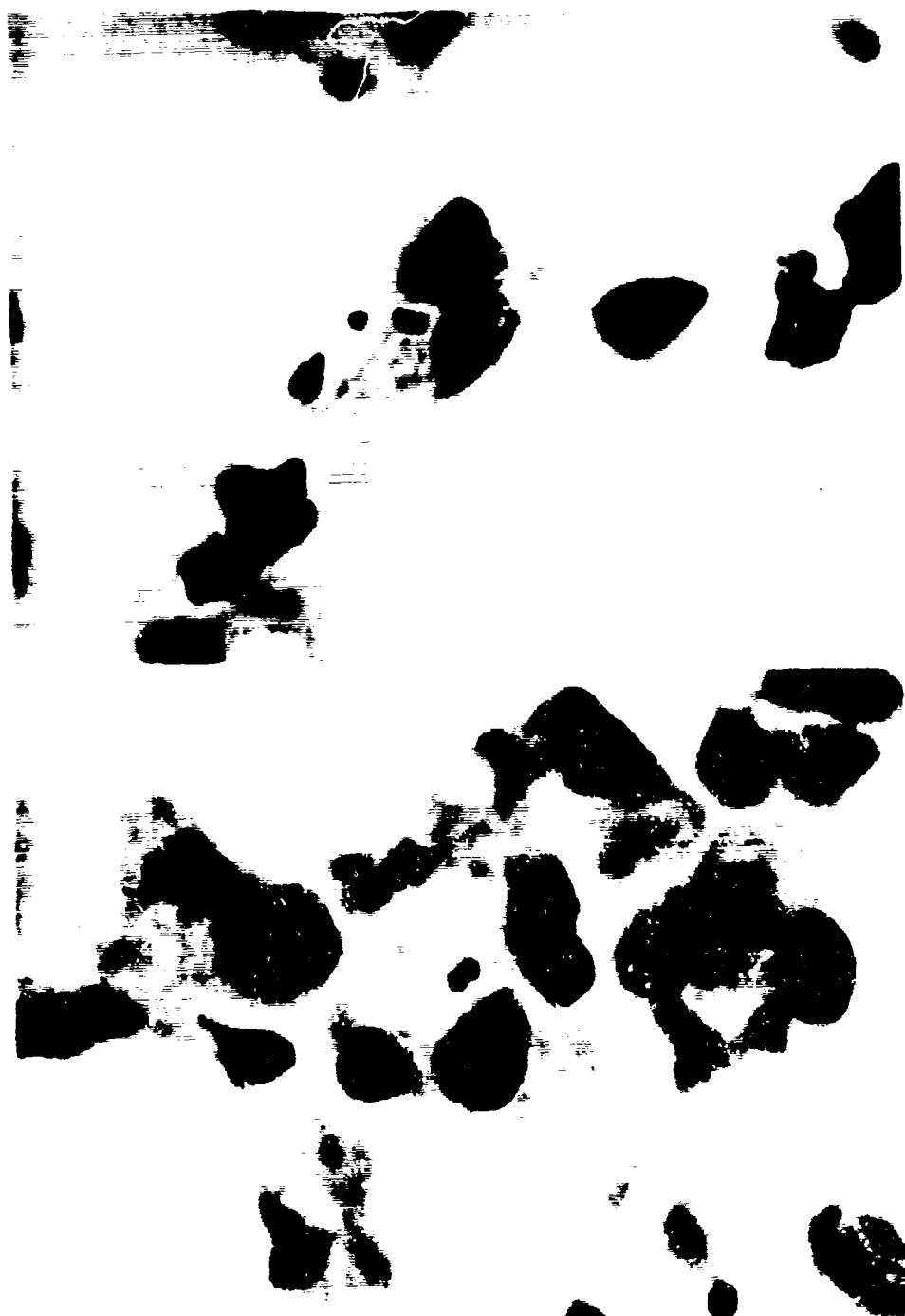
FIG. 3. Section of mouse lung immediately after 30 min of exposure to aerosol of Staphylococcus aureus stained by Maxallium-Goodpasture stain. × 2,500. In the upper photograph, staphylococci appear to have been ingested by an alveolar septal cell. In the lower, staphylococci may be in a mononuclear macrophage in the alveolar septum. see reference 6.
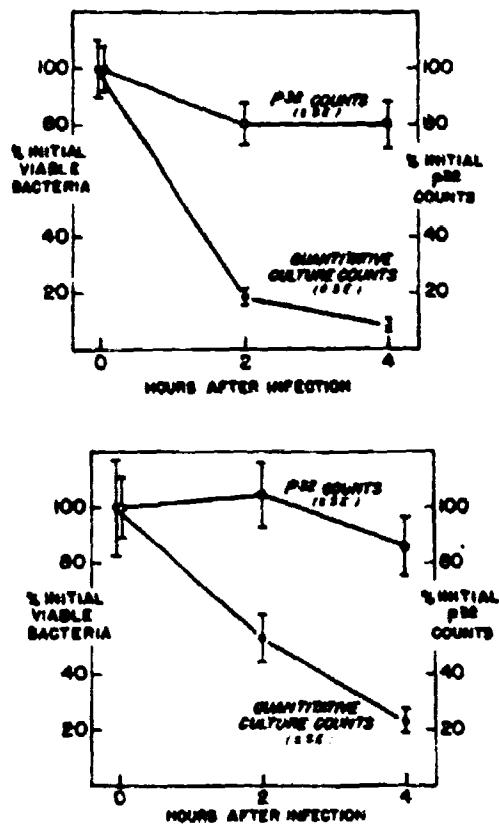
FIG. 4. *Clearance of* P^{32}-*labeled Staphylococcus aureus and Proteus mirabilis from murine lung. The change in number of viable organisms is compared with the change in radioactivity in the lung* (6).

ardized conditions could be obtained whereby a suspension of staphylococci could be delivered into the chamber in sufficient concentration that mice exposed to the aerosol for 30 min and sacrificed immediately thereafter were found to contain in their lungs approximately 50,000 viable units of staphylococci capable of producing colonies. When the lungs were cultured at regular intervals after exposure to the aerosol, the bacteria were found to have become nonviable in exponential fashion, so that within 4 hr approximately 85% of the bacteria could no longer be detected, and within 6 hr all but a few per cent had become nonviable. What was even more striking was the extraordinary reproducibility of the method, as seen by the small standard errors of the mean bacterial counts obtained at various time intervals after infection by aerosol (Fig. 1). The rates of disappearance of the bacterial particles were consistent with the anticipated rate of disappearance

of any particles of this size, but as is well known, particles of this size tend not to impinge on the bronchial mucosa, and so the implication was clear that most of the disappearance of the bacteria was likely to be due to cellular systems or other antibacterial systems operating below the tertiary bronchi, beyond the level at which the mucociliary apparatus is active.

Clearance by the Alveolar Macrophage System

To test the implication that bacterial killing occurred primarily in the deeper portions of the lung, two experiments were conducted (6). In the first, fluorescein-labeled antibody was used to detect bacterial antigen in the lungs of mice that had been exposed to the aerosols, and invariably bacterial antigen was found in the epithelial cells lining the alveoli (Fig. 2). Occasionally, relatively intact bacteria could be found in these alveolar-lining cells (Fig. 3). When the bacteria were labeled with radioactive phosphorus and their fate was studied, it was found (Fig. 4) that, when approximately 85% of viability had disappeared, radioactivity had declined by only about 20%. Thus, the decline in viability was not due to transport of the bacteria away from the alveoli, as evidenced by the retention of radioactivity and the observation of bacterial antigen in the alveolar-lining cells. Only a small minority of the bacterial population could have been transported away during the time of maximal killing. The majority of the bacterial cells were destroyed in situ, and the alveolar macrophage system clearly seemed to be the principal agency for such removal.

Role of Bacterial Species

That the bacterial species is an important variable in the process of removal was demonstrated by subsequent studies (5) in which the rates of removal of a strain of *Proteus mirabilis*, one of *Staphylococcus aureus*, and one of *S. albus* were studied under comparable conditions (Fig. 5). *S. albus* was removed most rapidly, *S. aureus* somewhat more slowly, and the strain of *Proteus* still more slowly. More recently, observations have been made with a pathogenic strain of *Pasteurella* that frequently produces pneumonia in mice and, as might be expected, clearance of this organism in some, but not all, animals is slower than that of *Proteus*, and occasionally clearance is completely reversed and bacterial proliferation is observed.

The wide variation in clearance of aerosolized bacteria in relation to species suggests that simple mechanical factors, such as the action of the mucociliary apparatus, are an unlikely basis for the antibacterial action, since it seems unlikely

Hr after Exposure to Aerosol
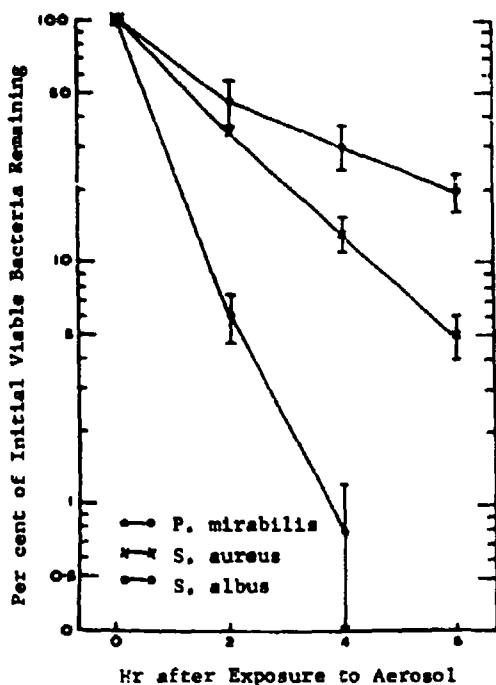
FIG. 5. *Clearance of Proteus mirabilis, Staphylococcus aureus, and S. albus by the normal mouse lung* (5).

FIG. 6. *Relative effects of ethyl alcohol, hypoxia, and cold on the clearance of Staphylococcus albus, S. aureus, and P. mirabilis by the normal mouse lung. Although in general these circumstances delayed bacterial clearance, the effect of hypoxia in the case of Proteus was negligible. It is also noteworthy that, in the case of Proteus, ethyl alcohol sufficiently depressed bacterial clearance to allow bacterial multiplication to occur* (5).

that a mechanical system would show such a wide range of effectiveness against different species of similar siz° range. However, such differences in antibacterial activity against different bacterial species are well known in phagocytic systems (2, 14). The observations also indicate that, since the methods of clearance of different bacterial species may be different from one another in a given host, circumstances that alter the rate of clearance may permit one or another species to multiply instead of being cleared.

BACTERIAL CLEARANCE AND ENVIRONMENTAL AND METABOLIC DISTURBANCES

To investigate the relationship of a variety of metabolic events to the clearing mechanism, experimental animals were exposed to the aerosol of appropriate bacteria and then immediately exposed to a metabolic circumstance that might be expected to alter the rate of bacterial clearance (4). It was found (Fig. 6) that hypoxia equivalent to an altitude of 10,000 ft was sufficient to slow significantly the rate of bacterial clearing, and this effect could also be obtained with diminished oxygen tensions at sea level pressures. Ethyl alcohol inhibited bacterial clearing by the lung and did so in direct relationship to the dose of ethyl alcohol

administered. Furthermore, the administration of oxygen to the intoxicated animals did not correct the defect. The latter experiment was performed because of the possibility that ethyl alcohol may have depressed respiration and thereby brought about depression of bacterial clearance. Acute starvation for 24 hr was associated with depressed clearance of bacteria by the lung, and once again the degree of depression of clearance was directly related to the amount of weight lost. In retrospect, however, the latter effect may not be entirely due to the weight loss itself, but may be related to such accompanying metabolic disturbances as acidosis (see below). Cortisol also depressed bacterial clearance significantly.

When the effects of the metabolic agents that inhibited bacterial clearance were tested in animals that had received different microorganisms in the aerosol, it was apparent that not all of the metabolically induced suppression of clearance was uniform regardless of species (5). For example, the clearance of staphylococci was only partly depressed by ethyl alcohol, but the clearance of *P. mirabilis* was completely inhibited and multiplication of the organism occurred. Thus, under conditions of ethyl alcohol intoxication,

with all three species of bacteria present in the lung, it might be expected that *Proteus* would emerge as the most likely organism to produce pulmonary infection. On the other hand, whereas hypoxia markedly inhibited the clearance of staphylococci, there was no depression of clearance of *Proteus* in the hypoxic animals. Presumably an oxygen-dependent system in the alveolar macrophage is operative against certain bacteria such as staphylococci, but not against other organisms, such as *Proteus*. Once again there are indications that specific environmental conditions in the presence of a mixed bacterial flora may favor the emergence of one or another bacterial species from the mixture. In vitro, phagocytosis by alveolar macrophages is depressed when oxygen tension is reduced (13).

Bacterial Clearance and Viral Infection

Another clinical circumstance in which pulmonary bacterial infection has been involved has been the presence of a precedent viral infection. Sellers and co-workers (15) demonstrated that in mice infected with influenza virus the intranasal insufflation of staphylococci was followed by virtually no clearance of the organisms by the lung, whereas in animals not infected with the influenza virus, the staphylococci were readily cleared by the murine lung. Detailed studies of this phenomenon have indicated (Fig. 7) that clearance of inhaled staphylococci is inhibited in the presence of a viral infection. However, quite unexpectedly it was found that the time of maximal inhibition of bacterial clearance by the virus-infected lung was toward the end of the 1st week after the induction of the viral infection. With relatively small inocula of virus, no inhibition of bacterial clearance was observed in the first 5 to 6 days after induction of the viral infection, even though the peak of viral multiplication had been reached by approximately 48 hr. On the other hand, distinct and striking inhibition lasting for 1 to 3 days was observed toward the end of the 1st week at a time when viral titers were falling rapidly. Precisely why this unusual time sequence should occur is a matter for future study. It is noteworthy, however, that, clinically, bacterial infections as superimposed complications of viral infections often appear about 1 week after the initial viral infection.

Previous bacterial infections with the same species seem not to inhibit function of the alveolar clearing mechanism very much, as might be expected from consideration of the relative numbers of bacteria inhaled in relation to the large numbers of alveolar cells available. Thus, when mice were exposed to an aerosol of staphylococci on succeeding days for 1 week, the rate of clear-
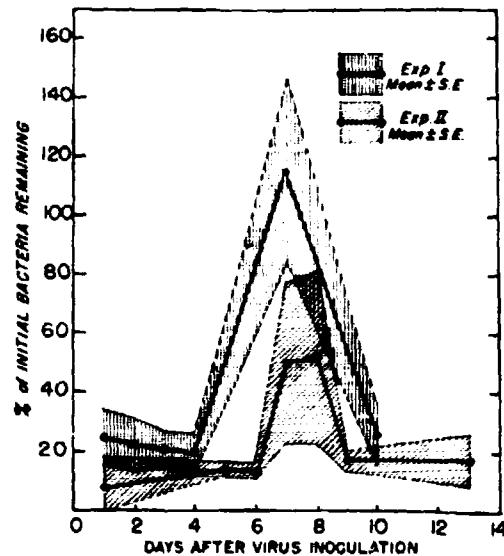
FIG. 7. *Effect of influenza virus infection on clearance of Staphylococcus aureus by the murine lung. The results of two separate experiments are given and plotted as the mean number of bacteria remaining after 4 hr, plotted as the per cent of the initial bacteria. The influenza virus infection was induced with 0.3 LD$_{50}$ dose.*

ance was not different after seven successive exposures than after the initial exposure.

Bacterial Clearance and Pulmonary Injury

At first glance it might appear that any injury to the lung would be associated with diminished bacterial clearance. However, the widespread nature of the alveolar system might also suggest that focal anatomic lesions would not inactivate a sufficiently large percentage of available cells to inhibit measurably bacterial clearance, except as the anatomic lesions became overwhelmingly severe. The latter of these two points of view seems to be the correct one. Goldstein (*unpublished data*) has produced silicosis experimentally by the intratracheal administration of silica suspensions, and severe coalescent disease was produced in the lungs of the animals. There was remarkably little effect on bacterial clearance, except perhaps in the terminal stages of the silicotic disease, when a variety of other metabolic consequences of severe pulmonary disease also begin to operate.

Bacterial Clearance and Tobacco Smoke

Most recently, an additional effect on pulmonary bacterial clearance by a particle of major

public health importance has been observed. It was initially observed by Laurenzi et al. (9) that mice exposed to tobacco smoke suffered inhibition of pulmonary bacterial clearance. More recently, it has been observed by Green and Carolin (*unpublished data*) that the addition of tobacco smoke to cultures of pulmonary macrophages rapidly altered the capacity of the macrophages to cling to the culture flask and greatly diminished the killing power of these cells for added staphylococci. The nature of the agent in tobacco smoke that produces this effect is not yet clear, but the substance is water-soluble and affects macrophage function quantitatively.

A useful methodological innovation that has come from these studies is a consequence of the earlier demonstrations that radio-labeled bacteria may be killed quite rapidly by the lung, but most of the label is readily recovered from the lung after 4 hr, when most of the bacteria are nonviable. In consequence of this observation, it has been possible to study rates of clearance in individual animals rather than in groups of animals, and to do so with considerable precision. It is only necessary to expose animals to radio-labeled bacteria of known specific activity and, after a given period of time, to count the radioactivity and the viability in the homogenates of the lungs. The radioactivity will afford an approximation of the total number of bacteria deposited in the lung, and the direct bacterial counts will indicate residual viability. From these data, the degree of killing can be estimated.

The method has added substantially to the precision of study of the pulmonary antibacterial system, and has made the standard errors of respective points smaller still. Even more important, it has permitted the study of clearance in individual animals and thus has greatly increased the efficiency of the experimental work. Finally, the method offers some hope that it can be adapted to the study of clearance mechanisms in the human being.

Bacterial Clearance and Renal Failure and Acidosis

A recent insight into another major metabolic circumstance that has been clinically associated with apparently increased susceptibility to pulmonary infection has come from the observation that nephrectomized animals or animals whose kidney

FIG. 8. *Upper portion of the figure indicates the numbers of colonies of Candida albicans in kidneys and lungs of mice after inoculation, and these are related to the levels of blood-urea nitrogen in these animals as well as the per cent clearance of Staphylococcus aureus observed 4 hr after completion of exposure to the aerosol. When numbers of Candida were highest in the lung but blood-urea nitrogen levels were relatively low, there was no significant effect on pulmonary bacterial clearance. As the renal lesion progressed and the numbers of organisms in the lung regressed, the blood-urea nitrogen rose,* *and there was a corresponding decrease in bacterial clearance. In the lower figure, the effect of nephrectomy on pulmonary bacterial clearance of S. aureus is demonstrated. Although the numbers of bacteria inhaled by each of the three groups is comparable, as evidenced by the comparable levels of P³² label in the bacteria, sham surgery slightly depressed bacterial clearance in 4 hr but nephrectomy markedly depressed bacterial clearance.*

function has been reduced in consequence of experimental candidiasis have decreased capacity to clear bacteria from their lungs (Fig. 8). It seems, from the present as yet incomplete analysis of the phenomenon, that the acidosis accompanying the uremic state in these animals is the primary source of the disturbed function of the macrophage system. The implication is clear that pulmonary macrophages harbor enzyme systems that are critical to phagocytosis or bacterial killing, and that are exceedingly sensitive to minute variations in pH. The search for such systems should be carried on forthwith.

SUMMARY

In summary, it is apparent that there is an in situ mechanism for clearing bacteria in the lung. This mechanism, which accounts for most of the antibacterial activity, appears to reside primarily in the pulmonary macrophage, and is relatively independent of the function of the mucociliary apparatus. Parenthetically, it has been observed that later in the course of events pulmonary macrophages laden with bacteria may become free and be carried upward by the mucociliary stream. The pulmonary macrophage system is peculiarly susceptible to a variety of metabolic situations, such as hypoxia, ethyl alcohol, acidosis, cortisol, tobacco smoke, and undoubtedly many others. The macrophage system responds differently to different bacterial species, and the metabolic circumstances that alter bacterial clearance do not affect clearance of each of the bacterial species in the same manner. Thus, a metabolic basis emerges whereby a single organism may emerge from a mixture of organisms as a pathogen under specific environmental circumstances. Viral infections inhibit the clearance of bacteria, but do so, strangely enough, after approximately 1 week of viral infection, and not at the time when viral replication is at its height. Multiple anatomic lesions, such as those accompanying diffuse silicosis, have relatively little effect on bacterial clearance, compared with the effects of the aforementioned metabolic states. Tobacco smoke has a water-soluble substance in it that inhibits the function of pulmonary macrophages.

How these observations relate to the genesis of chronic bacterial infection of the lung is only conjectural at present, but clearly the hypothesis can be stated that a variety of environmental circumstances may conspire to reduce slowly the capacity of the pulmonary macrophage to inhibit bacterial proliferation, and that then a chronic state of bacterial proliferation in the bronchial tree may result. It is conceivable that such a chronic state of bacterial habitation in the lung might be detected by appropriate methods long before manifest clinical pulmonary disease could be found, and in this sense the situation in which asymptomatic infections of the lung might be a precursor to chronic pulmonary infections could be analogous to a comparable situation in the urinary tract (7).

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Discussion

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Dr. Kass has reported highly reproducible measurements of the rate of clearance of staphylococci and other bacteria from the lungs of mice after aerosol inoculation. The aerosol particles were 1 to 3 μ in diameter, and the dose, given in a 30-min inhalation, was sufficiently large to permit recovery of at least 50,000 colony-forming units. Studies of lung sections with fluorescein-labeled antibody and by conventional staining methods revealed staphylococcal antigen and some intact bacteria in alveolar lining cells.

With this model, the effect of hypoxia, alcohol, starvation, and other influences was studied. In addition, it was shown that influenza virus infection interfered with the clearance of *Staphylococcus aureus* from the lung.

At this point, it is perhaps of interest to consider briefly the relationship of clearance of staphylococci by alveolar macrophages, referred to by Dr. Kass, with other clearance mechanisms. It is well appreciated at this conference that particles of the size used by Dr. Kass largely escape trapping in the nasopharynx and are carried to the lung. Here a large percentage are deposited, and the remainder are exhaled. Sites available for deposition are the alveoli, the alveolar ducts, respiratory bronchioles, and more proximal airway structures. Although gas exchange occurs quite readily between the tidal air and the alveoli through the layer of residual air in the alveoli, this is effected chiefly by the process of molecular diffusion. In contrast, only 10 to 20% of aerosol in tidal air actually exchanges with residual air with each breath, and molecular diffusion is not a significant factor with particles of the size presently under discussion. It is suggested, therefore, that substantial alveolar penetration will require prolonged periods of breathing

of aerosol, probably of the order of that used by Dr. Kass. With a few breaths, particles may be deposited in the lower respiratory tract proximal to the alveoli, and, with further breathing, the site of major deposition will progress peripherally, ultimately to the alveoli, as alveolar wash-in is completed. Parenthetically, I wonder if the slow movement of particles from tidal air to residual air may not be an important means of protection against toxic or infectious particulates in the environmental air.

Once deposited, particles may be removed from alveoli by alveolar macrophages and carried into pulmonary lymphatics. Some macrophages filled with particulates may also be discharged up the airway to the muco-ciliary blanket and then carried up the trachea. In the case of microorganisms which deposit in the respiratory bronchioles, the mode of disposition is not clear. Alveolar macrophages are apparently not available here, and the muco-ciliary blanket begins more proximally. Some studies, however, have described a hyperreactivity of respiratory bronchiolar lining cells which may be a special means of protection in this area. The small volume of lung airway represented by the tracheobronchial tree appears to be the best protected. Inhaled particles which deposit here are carried rapidly up to the posterior pharynx by the muco-ciliary mechanism, where they may be expelled or swallowed.

At present, I know of no studies which adequately describe relative degrees of deposition of small particles in peripheral lung areas in relation to the duration of exposure to small-particle aerosol. I believe the question to be of importance, since, if the foregoing concept is correct, it would be possible to deposit small-particle aerosol in

sites other than the alveoli, and mechanisms other than alveolar macrophages would be called forth to clear them from the lung. Instances of this sort may ı gularly occur in the natural spread of airborne infection.

Evidence for significant deposition of small particles at sites other than the alveoli is found in the work of McGavran et al. (4), who observed that pulmonary lesions of psittacosis in monkeys, after small-particle aerosol inoculation, developed around foci in respiratory bronchioles and that none were found developing around alveoli. These findings cannot be considered proof, however, since lesions develop a considerable time after inoculation, and a number of factors could influence the site of development of infection during this period.

I was especially interested in Dr. Kass' report that clearance of staphylococci from the lung was impaired in the presence of influenza virus infection, but only after it had progressed for 6 to 8 days. As he suggested, this coincides in time with the occurrence of some human cases of bacterial pneumonia complicating influenza. Harford et al. (2) in 1948 showed a similar result with pneumococci. In their studies, instilled pneumococci multiplied rapidly in mice during the 5th to 6th day of viral influenza, leading to pneumonia and death. Gerone et al. (1) in 1957, in similar experiments, found a rapid increase in pneumococci in the lung and high mortality in mice given bacterial challenge 6 to 9 days after influenza virus PR8 inoculation, but bacterial challenges given before this time were without effect. Although Kass did not report on mortality, it seems likely that an appreciable occurrence of pneumonia and mortality might have resulted in his studies.

Apparently quite distinct from the foregoing was the observation by Janssen, Chappell, and Gerone (3) that guinea pigs given S. aureus at the time of inoculation with influenza virus showed a high mortality within 48 hr. Influenza virus or Staphylococcus alone in the same doses had no effect. The effect was shown to be dependent on live influenza virus, and it did not occur in animals with influenza antibody produced by prior challenge. On the other hand, killed staphylococci served as well as live cultures in causing death.

Animals dying of this synergistic combination howed pulmonary consolidation. However, staphylococci could not be cultured from the lungs (in animals given live cultures), although influenza virus was present in high titer. One is tempted to compare these results with the occasional case of rapidly fatal human influenza in which no evidence of bacterial pneumonia is found. Thus, there may be two forms of interaction of staphylococci and influenza virus in animal infection which have a counterpart in natural human illness, i.e., bacterial superinfection late in the course of influenza (referred to by Kass), and an early, often fatal influenza, apparently unrelated to bacterial infection, but conceivably contributed to by constituents of killed staphylococci (referred to by Janssen et al. (3)) which have remained in the lung.

Dr. Kass' studies of metabolic and other factors which influence lung clearance are of great interest. If the relationship of lung clearance of microorganisms to the pathogenesis of pulmonary infection can be precisely defined, this model could serve an extremely useful purpose in attempts to identify mechanisms of susceptibility and resistance to infection. It would be highly desirable to extend some of these techniques to man, if the safety of the methods could be assured, but the problem is technically an imposing one.

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Airborne Disease and the Upper Respiratory Tract

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INTRODUCTION

Studies of airborne infection have been largely directed at the identification and nature of responsible microorganisms, epidemiology, host immunological defenses, and antibacterial or antiviral drugs. A relatively small effort has involved the possible role of the respiratory mucosa and the nasal passages in the defense against airborne infection. As a result, although a highly sophisticated body of knowledge has accumulated in the former fields, we do not yet know whether the upper respiratory tract constitutes a useful defense against airborne disease, is of no use, or may possibly be a detrimental influence. G. W. Wright pointed out this fact at the first Airborne Infection Conference five years ago (85), in saying, "One might well wonder whether the nose and nasopharynx are more harmful than helpful with regard to infections of the lower air passages."

The long-term objective of the work to be reported in this paper is to discover what part the upper respiratory passages and the respiratory mucosa play in defense against airborne disease and what factors influence their function.

It is conceivable that the nasal passage in man is merely a vestigial remnant of a once highly effective olfactory organ (37, 56) (Fig. 1). Yet, anthropological studies show that evolutionary changes have resulted in nasal dimensions which vary with climate demands (18), thus suggesting that, in man's development, the nose has not entirely regressed to a useless ornament.

On the one hand, available evidence indicates that the nose is less effective in humans than in other mammals as a filter for particles in the inspired air (3, 9, 19, 58), that particles carrying infectious organisms are not only small enough to pass through the nose but also to pass through the

tracheobronchial tree into the alveoli (17, 28, 42, 53, 76), and that many patients survive for years while breathing through a tracheotomy (31, 61). Unfortunately, no really adequate study has been done on such patients to determine the effect of tracheotomy breathing on airborne infection.

On the other hand, it is well known that, during the first few days after tracheotomy, pulmonary infections are common and often severe; some evidence from experimental induction of respiratory infection suggests that the nose may serve to protect the lower respiratory tract (13); clinical experience suggests that a diseased nasal passage is seldom found without concomitant lower respiratory symptoms, and, in mucoviscidosis, the abnormal function of mucous membranes seems the most obvious link with the susceptibility of these patients to frequent and severe respiratory infection. At least one study has described a relationship between the effectiveness of the nasal filter and the incidence of silicosis (45).

All of this leads to the conclusion that the role of the upper respiratory tract is still in doubt and requires further investigation.

ANATOMY OF THE UPPER RESPIRATORY TRACT

It will be helpful if workers in this field will agree upon clear-cut definitions of terms. The upper respiratory tract is that part of the air passages which extends from the larynx to the nostrils and to the lips, including the Eustachian tubes and the paranasal sinuses (Fig. 2).

This may be divided into the nasal passage extending from the mucocutaneous junction at the nostrils to the upper border of the soft palate (including the paranasal sinuses), the nasopharynx from the posterior nasal passage downward to the lower free border of the soft palate (including the Eustachian tubes), the mouth extending from the lips backward to the soft palate,

FIG. 1. *Upper respiratory tract of the Indian barking deer showing the relation between the epiglottis and the palate, assuring nasal air flow even when the mouth is open. This relationship exists in many mammals but not in man. From V. Negus, Comparative Anatomy and Physiology of the Nose and Paranasal Sinuses. E. & S. Livingstone, Ltd., Edinburgh, 1958, with the kind permission of the author and the publishers.*
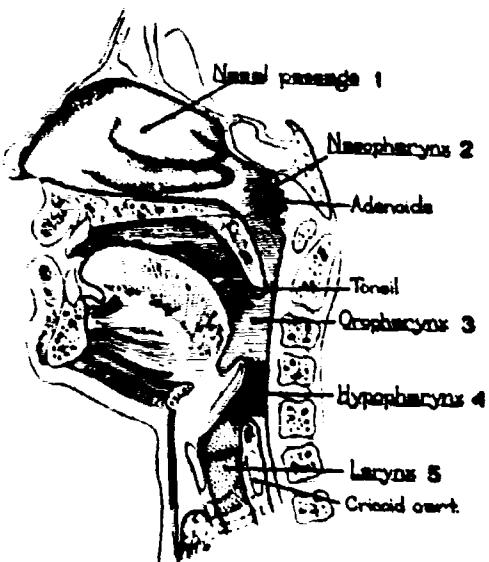
FIG. 2. *Diagram of the upper respiratory tract showing the anatomical divisions suggested in the text. The mouth is included in the oropharynx. The lateral extensions of the hypopharynx downward on either side of the larynx are not shown.*

FIG. 3. *Nasal airway. The cartilaginous and bony structures (horizontal hatching) fix the maximal dimensions of this portion of the airway, and the overlying vascular network and mucosa more or less narrow the passage according to their state of congestion. Communication with maxillary antra through middle meati is usually a thinner partition than shown here. Air passage is shown in solid black.*

the oropharynx extending downward from the free border of the soft palate to the epiglottis, the hypopharynx from the tip of the epiglottis downward into the pyriform sinuses laterally and to the aryepiglottic folds medially, and the larynx extending from the aryepiglottic folds down through the cricoid ring.

The dimensions of the nasal passage are relatively fixed by their cartilaginous and bony framework, but vary in width according to the thickness of the lining mucous membranes (Fig. 3). The nostril hairs placed at the entrance to the nose may play a part in air flow and aerosol distribution in the air stream. From the nostril to posterior nasopharynx in the adult is 8 to 11 cm and from the nasal floor to cribriform plate is 4.5 to 5 cm. The dimensions of the nasopharynx vary with the size of the adenoid mass above and with the position of the soft palate below. The mouth will vary from a broad passage when the tongue and palate are widely separated to a closed space when they are approximated. The oropharynx will be narrow or wide as the tongue is moved backward or forward and will also be affected by the size of the tonsils. The hypopharynx is relatively constant except during deglutition. The laryngeal airway is effected by both deglutition and by the motion of the true and false vocal cords.

The mucous membrane in the respiratory tract is ciliated columnar epithelium from a line just posterior to the anterior ends of the turbinates back into the nasopharynx, except for the olfactory area, but including the lining of the paranasal sinuses. In the nasopharynx there is a transition from ciliated columnar to transitional and then squamous epithelium. Over the adenoid tissue, there are alternating patches of squamous and ciliated columnar, but the crypts are entirely lined with keratinizing squamous. The common belief that adenoid tissue does not exist in the normal adult is untrue. At least small amounts of lymphoid tissue with crypts can be found in man of all ages (2). The Eustachian tubes are also lined with ciliated mucosa. The remainder of the upper respiratory epithelium with the exception of the ciliated posterior wall of the larynx is squamous.

Within the nasal passage, the vascular bed is so rich and subject to such wide changes in dilatation that it is commonly referred to as erectile tissue. There is also a particularly rich vascular supply within the tonsillar and adenoid tissue.

Goblet cells and mucosal glands supply a continuous carpet of mucus which lines the entire upper respiratory tract. This mucus is kept continually on the move by ciliary activity and swallowing from every point in the respiratory tract toward the hypopharynx and thence to the esophagus.

The ciliated mucosa of the trachea extends upward through the posterior commissure of the larynx; but motion of mucus through other portions of the larynx above the cricoid is largely determined by cough or "throat clearing" (Fig. 4).

The paranasal sinuses consist of a group of air spaces in the bones of the face communicating with the nasal air stream through small openings, and, in the case of the frontal sinuses, through long nasofrontal ducts. The direction of the mucociliary stream in all of the sinuses is toward the nasal passage.

The Eustachian tubes are normally closed channels connecting the nasopharynx with the air spaces of the middle ear. Here also ciliary activity moves mucus toward the nasopharynx. These tubes normally open during swallowing and yawning. They are essential for the maintenance of normal pressure within the middle ears and are of importance in respiratory infection as the chief passage through which pathogenic microorganisms gain access to the middle ears.

Whether or not the upper respiratory tract is an important factor in the defense against inhaled materials, it is certainly an important contributor to the adjustment of the temperature and water vapor content of inspired air and probably is
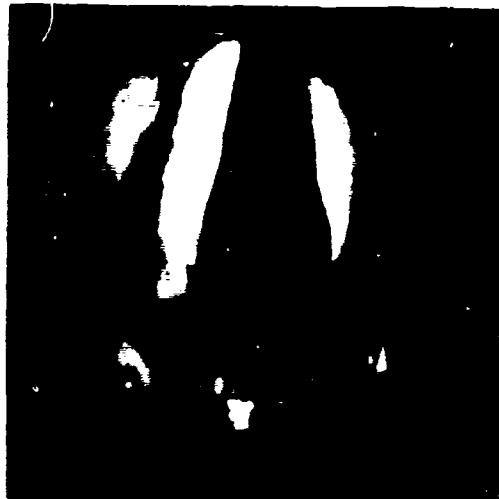
FIG. 4. *Photograph of the human glottis seen from above. Below is the posterior commissure through which the mucociliary stream must pass. The vocal cords are not ciliated. With the kind permission of Paul Holinger, Chicago, Ill.*

important in the maintenance of normal body temperature and water (12, 15, 22, 32, 38, 51, 56–58, 72, 73).

Although it is true that these functions can be taken over by the mucosa lining the tracheobronchial tree, observation of patients with tracheotomy (even long term) indicates that the nasal passage is best suited for this purpose. Normally, with nasal breathing, air temperature is close to body temperature, and the air is near to saturation with water vapor by the time it reaches the hypopharynx (63).

UPPER AIRWAYS, NASAL OR OROPHARYNGEAL

Because the narrow nasal passage is the place of greatest resistance to air flow (24), when the ventilatory demand rises beyond a certain point, one resorts to mouth breathing. Under these circumstances, the tongue is depressed and the palate raised, providing a wide airway with minimal resistance to flow. What work load creates this demand and how much individual variation there is has not been determined.

In like manner, when the nasal airway is sufficiently reduced by physiological alterations in mucosal and submucosal vasculature or by pathological processes, even quiet breathing may occur through the mouth; but in this case the oral airway consists of a narrow slit between tongue and palate. Here there is wide individual variation. Some patients will complain of nasal obstruction

FIG. 5. *Pneumotachogram during nasal breathing (left), followed by talking, with brief inspiration in center. Note air flow during this inspiration is more than twice that in resting breathing. With the permission of the Editor, Inhaled Particles and Vapours II (in press), Pergamon Press, Inc., New York.*

FIG. 7. *Tomograms of the upper airways. To the left, lateral view, and, to the right, anterior view. Compare with Fig. 3 and 8. With the permission of the Editor, Handbook of Physiology, vol., 1 Respiration, American Physiological Society, Washington, D. C.*

FIG. 6. *Oropharyngeal airway during deep breathing (A) and during inspiration between conversational phrases (B). Lips may be seen to the left. Note narrow airway between tongue and palate in (B). From cine-fluorograph, with thanks to Sue McCarty and Martin Donner, Johns Hopkins Hospital. With the permission of the Editor, Inhaled Particles and Vapours II (in press), Pergamon Press, Inc., New York.*

FIG. 8. *(A) Obverse of cast of nasal passage taken at autopsy; (B) Model constructed from same. Nostril is to the left. With the permission of the Editor, Inhaled Particles and Vapours (in press), Pergamon Press, Inc., New York.*

when their measured resistance to air flow is less than that found in others who are unaware of any difficulty in nasal breathing.

Movement of air in and out of the paranasal sinuses and middle ears occurs as a result of respiratory cycle pressure changes when these spaces are in free communication with the moving air stream, and as a result of gas absorption from these spaces when this communication is periodi-
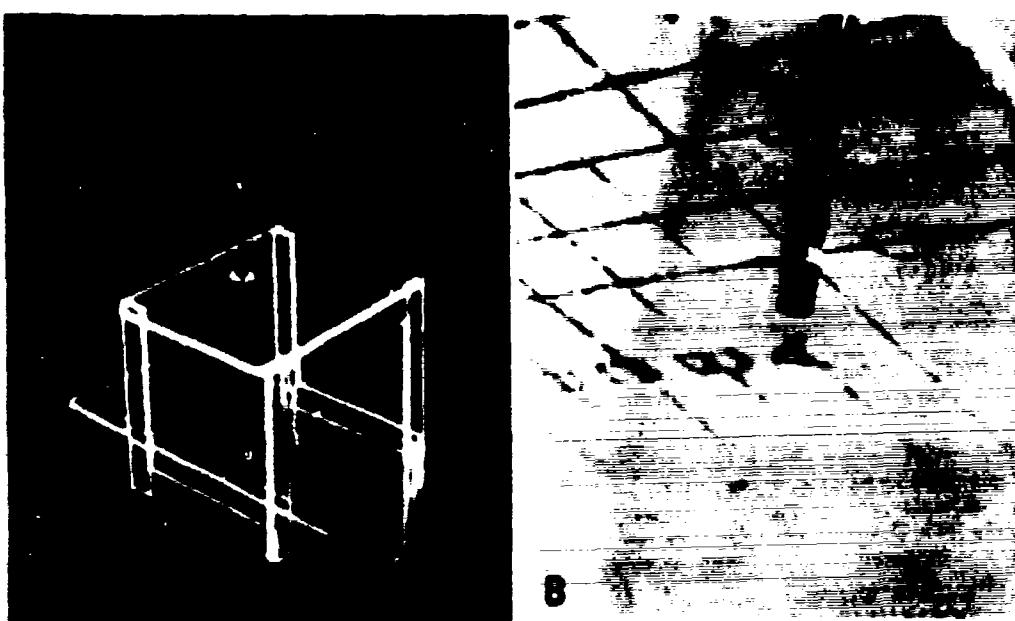
FIG. 9. (A) *Angle meter and grid (representing sliding nasal septum) used with models as in Fig. 8B.* (B) *Detail of tip of meter, showing three openings projecting into airstream. With the permission of the Editor, Inhaled Particles and Vapours (in press), Pergamon Press, Inc., New York.*

cally interrupted. Under normal circumstances, the gas contained within the sinuses undergoes one full change every few hours (63). Under unusual circumstances, especially those associated with marked atmospheric pressure changes, ventilation of these paranasal spaces may be much greater. Rahn calculated that in the Japanese women divers (Ama) the ventilation of each middle ear approximates 1,800 ml per day (67a).

During conversation, inspiration occurs through the mouth, but here again the oral airway is narrow. During singing, when it is necessary to fill the lungs quickly for long phrases, the oral airway is wide as in high ventilatory demands (Fig. 5 and 6).

If resistance to air flow is external, as with respiratory masks, nasal breathing continues until such resistance is extremely severe.

Although it is commonly recognized that nasal congestion sufficient to cause mouth breathing may be related to a multitude of internal and ernal environmental factors, these relations as emain generally undocumented. Such in- es probably include emotional stimuli, such s or sexual conflict, endocrine disturb- ch as hypothyroidism, and sudden inspired air temperature. Especially in motional stress, changes occur in all branes but are generally more

readily noticed and more pronounced in the nose (35, 49, 54, 60, 75, 84).

The function of the paranasal sinuses in man is open to question. It is clear that these air spaces provide protection for the brain against blows on the face. It seems likely that, in addition, they act as insulators and a source of mucous secretion to supplement the air-conditioning function of the nose (57, 58, 67).

CHARACTER OF UPPER RESPIRATORY AIR FLOW

The fate of inspired particulate matter depends upon the size and weight of the particle, the character of air flow, and the relationship between the moving air stream and the surfaces over which it passes. Since, within the nose the air stream is narrow (Fig. 7) and moves at a high linear velocity, and since turbulence is more likely to occur there than elsewhere in the respiratory tract, it is of importance that we understand the nature of air flow in the nasal passage and the factors which may significantly alter this flow (20, 38, 59, 66 74, 80).

Because of the difficulty in introducing measuring devices within the nose without interfering with the nasal air stream, models of the nasal cavity have been constructed from casts made at autopsy to permit measurement of simulated nasal air flow at all points (Fig. 8). These measurements
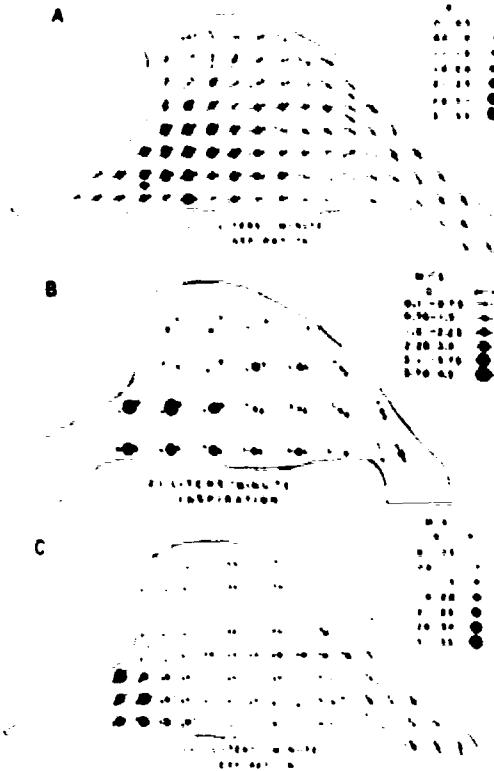
FIG. 10. *Patterns of air flow through one model. Arrows indicate direction of flow and circles indicate linear velocity. (A) Inspiration at 11 liters per min. (B) Inspiration at 21 liters per min. (C) Expiration at 11 liters per min. Note more diffuse distribution of flow in C. With the permission of the Editor, Inhaled Particles and Vapours (in press) Pergamon Press, Inc., New York.*

are made by use of an angle meter introduced into the stream through a simulated nasal septum which can be moved over the surface of the nose model (Fig. 9).

Such studies indicate a concentration of most of the moving air stream during inspiratory flow over a relatively small portion of the nasal surface. This may be useful in shunting the inspired air away from the unciliated olfactory area. It also may be useful when one is breathing noxious air, in that a large surface of neighboring mucosa remains unexposed and able to replenish altered or dried mucus in exposed areas (Fig. 10).

The path of the air stream remains remarkably constant in the face of changing flow from 1 to 100 liters per min. In the models studied thus far, as flow increases there appears to be a greater concentration of the moving air stream in its principal path along the middle meatus. In one model, this change occurred sharply at 16 liters per min, a flow which approximates the peak one might expect through one side of the nose in rapid nasal breathing (Fig. 11). In other models this sharp change has not been so evident, but a similar alteration in flow has been found in all (Fig. 12 and 13).

Changes in the nasal airway resulting from pathological conditions have also been studied in these models. Polyps, septal deviations, alterations at the nostril, enlarged adenoids, and generalized mucosal thickening have all been simulated. Alterations in the main nasal cavity, such as would result from polyps or septal deviations, seem to have the most effect on air flow patterns and might influence particle deposition or concentrate the air stream in small areas, thus producing an undue drying effect upon mucosa.

During expiratory flow, there is a more diffuse spread of the stream through the entire nasal passage including the olfactory area (Fig. 14 and 15). Maximal olfaction occurs just after a sniff when air may rapidly diffuse into the olfactory area.

Landahl has calculated the maximal linear velocity in the respiratory air stream to be about 2 meters per sec, and this occurs in the secondary bronchi (43). In our model studies, it is clear that peaks of at least 5 meters per sec occur briefly in the main stream of flow during quiet nasal breathing. Such velocities in a narrow curving air stream will surely influence the chances of particles contacting the surface.

If inspired particles are hygroscopic, upon entrance into the efficient humidification apparatus of the nose, they will increase in size. Theoretical considerations based on the behavior of particles in tubes or even in the experimental animal may be misleading and cannot substitute for measurements of what actually occurs in the human nose.

The relatively sharp bend of the air stream at the nasopharynx plus the fact that the main air stream at this point travels along the posterior wall increases the chance for impaction of particles on the adenoid tissue.

Studies of oropharyngeal and laryngeal air flow are needed to understand what role these portions of the respiratory tract may play in the fate of inhaled particles.

FATE OF PARTICLES DEPOSITED ON UPPER RESPIRATORY SURFACES

In collaboration with Henry Wagner, Jr., Betsy Bang, and James Langan, and with the advice of Anna Baetjer, three methods of following muco-
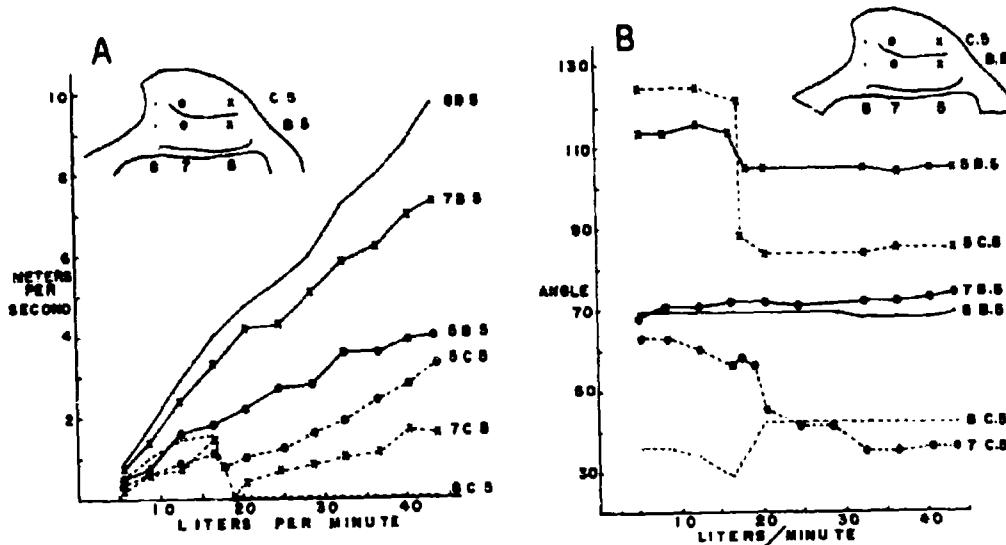
FIG. 11. Changes in linear velocity (A) and direction of flow (B) with increasing flows through model charted in Fig. 10 at points indicated in inset. Note sharp breaks in both charts at points above main flow paths at 16 liters per min. With the permission of the Editor, Inhaled Particles and Vapours (in press), Pergamon Press, Inc., New York.

ciliary clearance of particles have been explored one of which was combined with the use of a visible dye. For each technique, 0.02 to 0.1 ml of a saline solution containing 8 to 20 μc of radioactivity was injected with a microsyringe on the mucosal surface at the anterior nares. A head mirror and nasal speculum were used to assure placement of the material just behind the anterior end of the inferior turbinate. I^{131} was the isotope most frequently employed. In most studies, the isotope used labeled aggregates of human serum albumin (81), usually from 5 to 100 μ in diameter, but in one study (Fig. 17) 5 to 15 mμ (64, 65). In other studies, the isotope was in the form of a solution of sodium iodide, or a solution containing fluorescein mixed with the dye Sky Blue (dimethoxydiphenyl-diazo-bis-8-amino-1-naphthol-5,7-disulfonic acid) [C$_{34}$H$_{24}$N$_6$O$_{16}$S$_4$Na$_4$]. Subjects were given Lugol's solution by mouth prior to the study to block entrance of the I^{131} into the thyroid gland.

For the first method, immediately after placement of the isotope, the subject lay prone on a conventional scanning table with the head turned to one side (Fig. 16). A series of scans of the nasal area were then done as quickly as possible until the radioactivity was detected in the nasopharynx (Fig. 17). In most studies, the test was completed in 10 to 30 min, but in one study scanning was continued for 70 min to demonstrate retention in the anterior unciliated area (Fig. 18).

Such relatively long scans may provide important information. Both Hilding (33) and Macklin (50) pointed out the possible importance of small areas of poor clearance in the tracheobronchial tree in the role of carcinogenesis. Whether such areas regularly occur in the upper airways or whether they result from specific environmental circumstances is not known.

One study was done on a child with mucoviscoidosis (Fig. 19), but no other patients have been studied as yet.

For the second method, the subject was seated in a chair with a head rest and remained in this position throughout the test (Fig. 20). A double-channel collimated crystal scintillation detector was brought alongside the face and positioned so that the two channels pointe across the nasal passage, one just behind the point of injection, and the other 4 cm farther back. Radioactivity was then recorded at each point until the isotope had been carried backward past the second position (Fig. 21).

The third method (carried out in collaboration with Betsy Bang and James Langan) was similar to the second, except that a single detector was placed in front of the nose, pointed along the line of the nasal passage. Thus, as the isotope was carried backward, detected radioactivity fell in proportion to the square of the distance. In this series of studies, the Sky Blue was mixed with the isotope and looked for in the oropharynx about
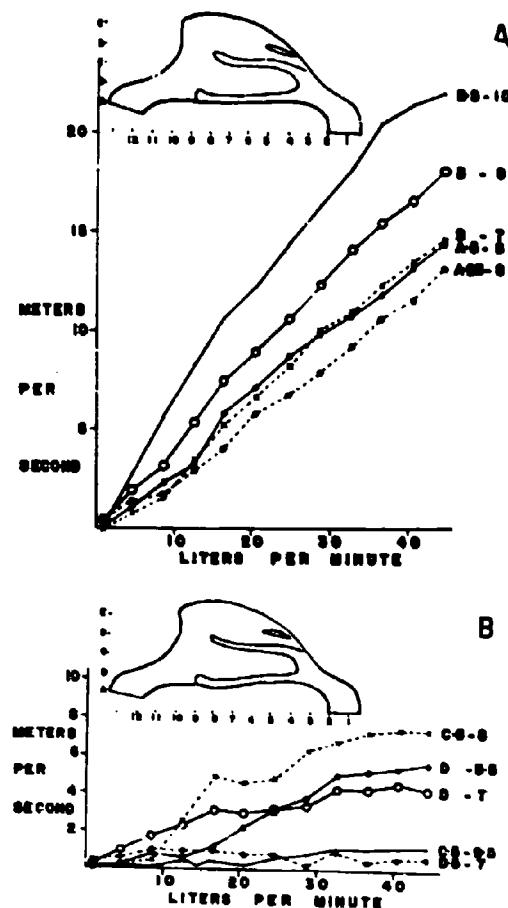
FIG. 12. *Changes in linear velocity with increasing flow in another model, at points in main stream (A) and at points away from main stream (B). Points are indicated on diagram. B·5 refers to the line halfway between (B) and (C), etc.*

once a minute. This dye, which is very clearly visible on mucosal surfaces, produced no unpleasant sensation in the subject, and appeared to have no unfavorable effect upon ciliary activity. In each subject, the appearance of the dye either at the edge of the soft palate or on the posterior pharynx coincided with a fall in radioactivity as detected in front of the nose. In most subjects, the visualization of the dye occurred just before the fall in detected radiation reached a plateau (Fig. 22).

The use of visible materials to study muco-ciliary activity has the advantage of simplicity (23, 68). Nevertheless, although the isotope technique demands complex equipment, it requires a minimum of cooperation on the part of the subject, gives a much more complete picture of the path of the mucociliary stream, allows one to de-
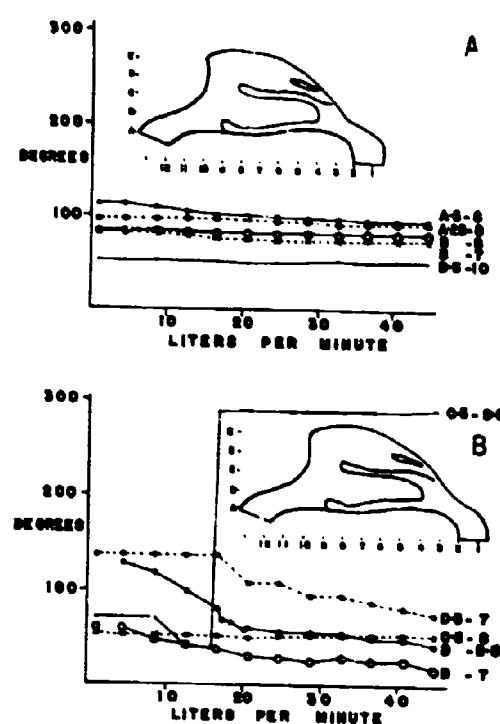
FIG. 13. *Changes in direction of flow with increasing flows, at points in main stream (A) and away from main stream (B) as in Fig. 12: 90° is horizontal to the floor of the nose.*
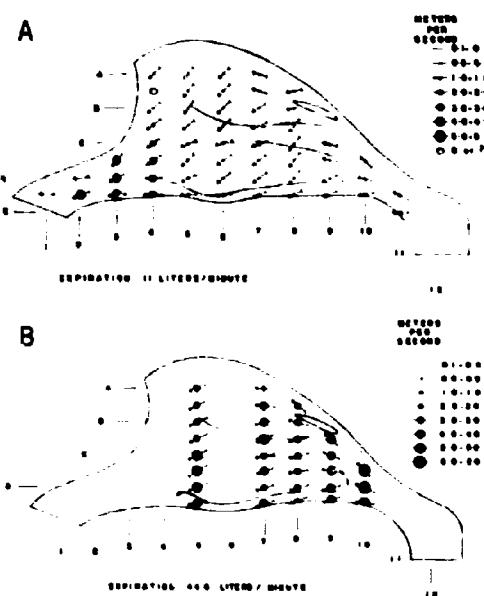
FIG. 14. *Patterns of flow in model charted in Fig. 12 and 13. (A) Expiratory flow at 11 liters per min and (B) at 44.6 liters per min. Note diffuse distribution of flow even in (B).*
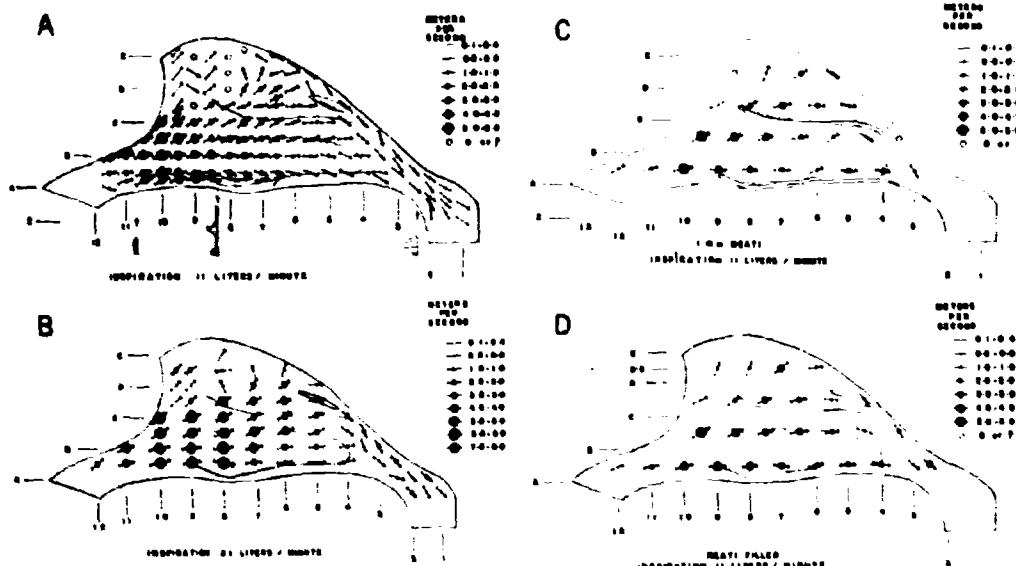
FIG. 15. *Patterns of inspiratory flow in model charted in Fig. 12, 13, and 14. Hatched areas in lower part of A indicate approximate cross section of airway. Letters and numbers refer to the grid locations as shown in charts in Fig. 11, 12, and 13. In C and D, the meati were narrowed (C) and filled (D) to simulate changes in airway to be expected from swollen mucosa.*

tect areas of retention, and permits study of portions of the respiratory tract not readily accessible to visual observation.

Thirty-six subjects have been studied in 64 tests thus far: 27 males and 9 females, ranging in age from 7 to 52 years, only one of whom had gross respiratory disease. This number is insufficient for the establishment of normal values; and studies of normal subjects in varied environmental circumstances and of patients are just beginning. There have been 23 scans, 28 studies with the double detector, and 13 with the single detector.

The mucociliary transport of surface materials in the human nose seems to occur at about the same average speed observed in previous studies of respiratory mucous membranes. It is clear, though, that this is not a uniform speed. Portions of a drop even as small as 0.02 ml may require 2 to 10 min to move 6 to 9 cm backward into the nasopharnyx, whereas other portions of the same drop may require 8 to 15 min to travel the same distance; still other portions (at the anterior unciliated area) undergo no detectable motion.

There is wide individual variation, with some normal subjects showing transport times two to three times faster than others. Not enough studies have been done to discover how much variability there is in a single normal subject from time to time, or how variations may be related to environmental or other influences.

FIG. 16. *Scan superimposed on skull radiogram for orientation. This scan is taken from series to left in Fig. 17 and is from same subject in Fig. 6 and 21 A. With the permission of the Editor, Arch. Environ. Health (64).*

DISCUSSION

It is believed that microorganisms are airborne in droplet nuclei 2 to 3 μ in diameter (70, 82). Although there are relatively few studies of nasal particle deposition in man (and in these studies there is not complete agreement), it seems likely that many particles smaller than 5 μ will penetrate the to lower respiratory tract (29, 30, 34, 45, 53).
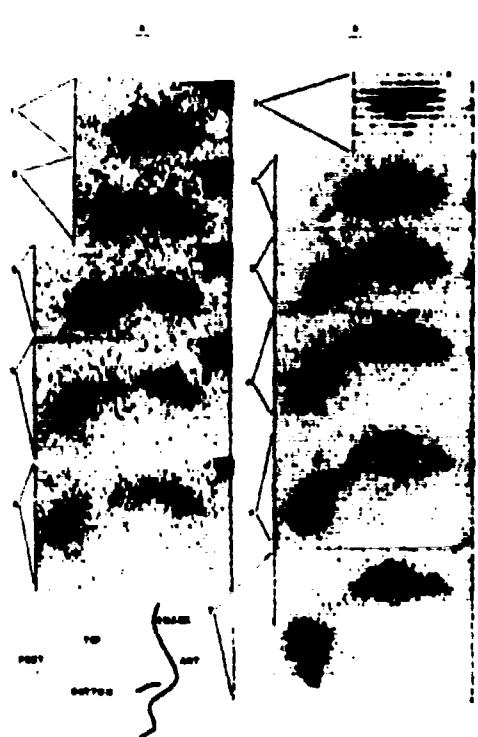
FIG. 17. *Series of nasal scans on 22-year-old normal female in left column and 52-year-old normal male to right. Times after injection reading from above downward, left: (1) 0.9 to 3.5, (2) 3.7 to 6.2, (3) 6.9 to 9.7, (4) shown in Fig. 16, (5) 13.7 to 17.9, (6) 19.7 to 24.9 min. On the right: (1) 1.2 to 2.5, (2) 3.4 to 5.7, (3) 6.3 to 9.7, (4) 9.6 to 13.2, (5) 14.6 to 16.0, (6) poor scan (omitted), (7) 29.5 to 34.7 min. With the permission of the Editor, Arch. Environ. Health (64).*

FIG. 18. *Series of nasal scans on same subject as shown in Fig. 17 (right) to illustrate retention of particles in anterior unciliated area. First scan (upper left), 0.2 to 1.5 min; ninth scan, 18.7 to 20.6 min; and last scan (lower right) 75 to 77 min after injection. With the permission of the Editor, Arch. Environ. Health (64).*

Three factors deserve further investigation in this connection: the possibility that droplet nuclei are hygroscopic and increase in size in the nose, the possibility that coagulation of particles may occur, and the effect of turbulence in the stream.

Since maximal exposure to airborne infection may occur in circumstances where one is indulging in animated conversation, the fate of particles in the oropharynx in these circumstances also deserves further study.

A recent report on the epidemiology of tuberculosis suggests the possibility that the nose may be an important defense. This report, and at least two other studies, indicate the likelihood that cross-infection has occurred between persons singing together, whereas infection did not occur between similar individuals sleeping in neighboring beds, sitting together in crowded classrooms or riding together on crowded buses (7, 36, 33, 78).

Two factors could combine to explain this interesting observation. The passage of air through the vibrating glottis may provide an excellent atomizer for the production of very small mucous particles. At the same time, since inspiration during singing consists of deep breaths through a wide open oropharynx and glottis, maximal opportunity for penetration of airborne particles into the depths of the lungs will result (Fig. 6).

In contrast, inspiration during conversation occurs through a narrow oral slit at relatively high linear velocity, a situation which could result in a filtration of particles similar to that normally found in the nose (Fig. 5 and 6).

If the upper respiratory tract plays a significant role in the removal of particles from the inspired air, the next question involves their fate once deposition has taken place (10, 11). Four possibilities are worthy of investigation: mucociliary clearance with dispatch through swallowing into the stomach, the passage of viable organisms through gastrointestinal mucosa, penetration through the mucous carpet into upper respiratory
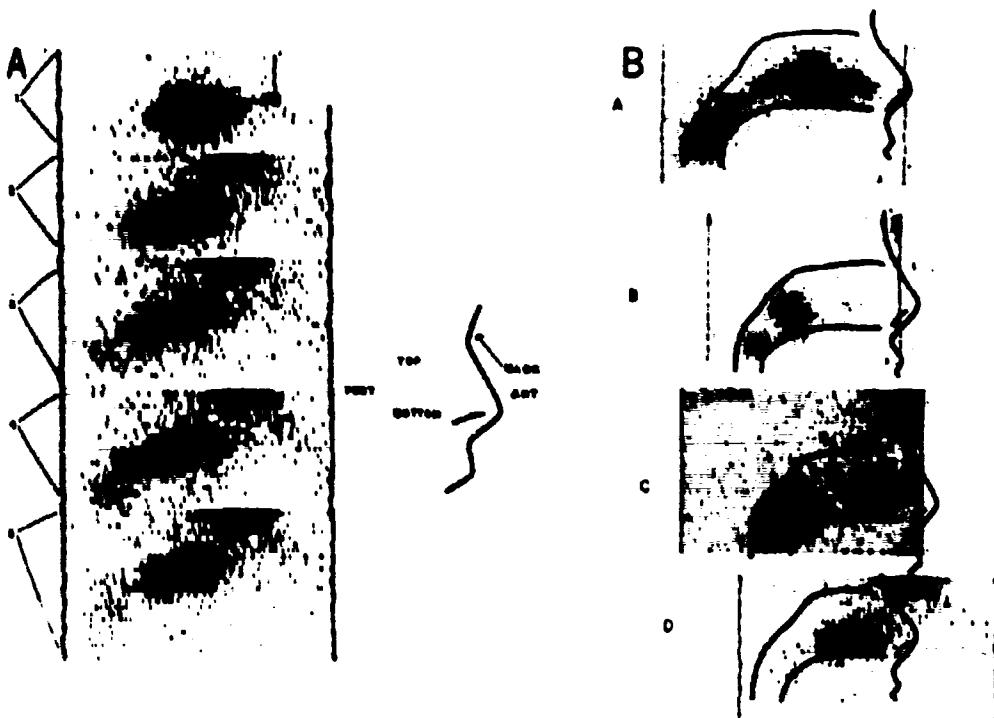
FIG. 19. (A) Series of nasal scans on 7-year-old boy suffering from mucoviscidosis. Times after injection, first scan (top), 0.9 to 2.6; last scan (bottom), 9.3 to 11.6 min. Note relatively poor clearing. (B) Scans at the 9th min from three normals (A, B, and C) and the child in A (D). With the permission of the Editor, Arch. Environ. Health (64).

mucosal cells, and deposition in the crypts of the adenoids.

Certainly, some organisms are deposited in the adenoids, but we know little about the factors which determine whether active infection ensues or whether the presence of pathogenic microorganisms in lymphoid crypts is a benign infestation leading to an opportunity for the body to thereby develop immunity to them (62).

It is evident that pathogenic bacteria may reside in the upper respiratory tract without producing signs or symptoms of disease (8, 26, 83) What enables such bacteria to institute active infection is not clear. It is conceivable that their deposition and residence, especially in the adenoid crypts, may be entirely innocuous, and that only when mucous membrane defenses are injured in some manner are such bacteria able to invade the body tissues and produce the signs and symptoms of infection.

Information is especially scarce regarding the chemical nature of respiratory-tract mucus. Mechanisms by which its water content and viscosity are varied to meet the changing demands of

our everyday environment are virtually unexplored. It has been established that antibodies are found in mucus, and it is possible that such antibodies may, on occasion, be more abundant and more effective against airborne infection than those which circulate in the blood stream (4, 14, 27, 46, 65, 79).

The method by which inhaled viruses pass through the moving mucous layer and gain entrance to surface cells remains unclear (79). It does seem that such penetration will be less likely to occur when the particle carrying the virus is kept rapidly on the move in the mucociliary stream. Stasis at any point in the stream would provide the needed opportunity for contact with cells, penetration, and infection. It should be remembered that any influence which slows the stream may lead to stasis. The more slowly the mucous stream moves, the longer it is exposed to the drying effect of the moving air, and the more likely such drying and the consequent rise in viscosity are to lead to the inability of the cilia to maintain mucous motion. Any factor acting directly either to impair ciliary activity or to in-
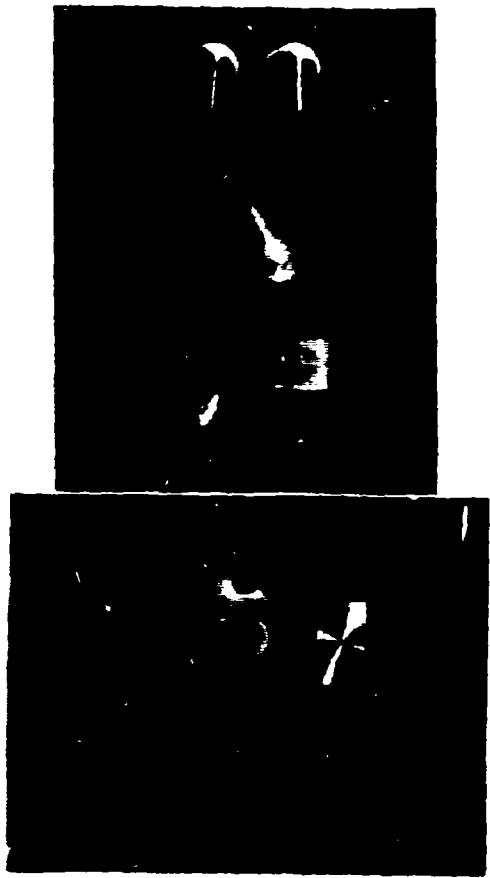
FIG. 20. (A) Double collimated crystal scintillation detector showing slits 4 cm apart. (B) Detector in place alongside subject's face. With the permission of the Editor, Arch. Environ. Health.

FIG. 21. Redrawn record from the double detector (A) and actual record (B). (A) From same subject illustrated in Fig. 6 and 17 (left). (B) From normal 35-year-old male. With the permission of the Editor, Arch. Environ. Health and Inhaled Particles and Vapours II (in press), Pergamon Press, Inc., New York.
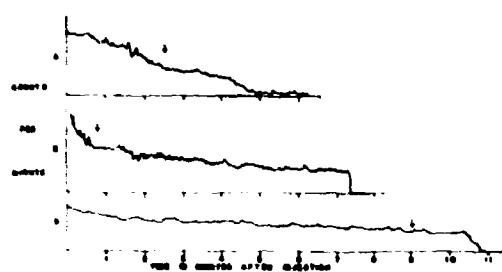
FIG. 22. Tracings from records obtained with single detector method: (A) 26-year-old normal male; (B) 36-year-old normal male; and (C) 3?-year-old normal male. In these records, the fall of radioactivity detected at probe in front of nose results from motion of isotope backward in mucociliary stream. Plateaus in (B) and (C) probably represent material retained in anterior unciliated area. Arrows indicate time at which dye became visible in posterior pharynx.

crease viscosity of mucus may thus reduce the effectiveness of respiratory mucosa, at least as an air conditioner and perhaps as a defense organ.

Although a great many studies have been directed at mucociliary activity and particle clearance, most of this work (owing to the paucity of techniques applicable to the human subject) has been done on in vitro mucosal strips or in the experimental animal (1, 5, 6, 16, 21, 23, 25, 41, 47, 68, 71). The use of radioactive tracer materials and apparatus for their external detection permits the study of mucociliary activity in living man (1, 25, 34, 55, 64, 65).

Although it is possible that many infectious organisms travel in the inspiratory air stream directly to the alveoli, there are broad gaps in our knowledge of particulate behavior in the airways and suggestive areas of disagreement (33, 39, 40,

52, 77). We cannot now state with certainty whether or not the upper respiratory tract is a barrier against airborne disease. We cannot know until we first understand normal human respiratory function (air flow and mucosal function) and what influences disturb it. Only then will it be be possible to search for correlations between such disturbed function and susceptibility to disease.

Many of the conclusions thus far drawn regarding the upper respiratory tract and mucous membrane function come from in vitro studies or the experimental animal. Although some of these conclusions are probably correct, it is time to check them with carefully controlled experiments on the human subject. This is especially true in regard to respiratory air flow and the fate of inhaled particles.

Clinical experience with respiratory infection has long suggested relationships between susceptibility and such factors as change of season, cold weather, allergies, exhaustion, emotional stress, etc. Studies of naturally occurring infections have not produced data leading to clear conclusions, perhaps because they have not included concomitant studies of mucosal function. Studies in the experimental induction of respiratory infection have in general failed to substantiate any of the relationships mentioned, but, instead, indicate that whether or not an individual exhibits signs and symptoms of infection is largely a matter of degree of exposure to, and immunity against, the infectious agent (10, 11, 12, 39, 60, 79).

Now and then, one does find evidence suggesting that susceptibility may vary with other factors, but the most suggestive evidence comes in children with mucoviscidosis. These children seem to have at least average capability for development of antibodies against infectious microorganisms, but, in spite of this, become infected frequently and tend to suffer from unusually severe infections. What is more significant is that their infections are almost exclusively respiratory. Here, the facts strongly indicate either that mucous membrane, when functioning normally, is a potent defense against airborne infection, or that there is some other now unknown factor involved.

To document any possible role of the upper respiratory tract or respiratory mucous membrane in general, we must be able to measure air flow and mucociliary activity in the normal human subject and in the patient before and during respiratory disease. Whether or not naturally occurring respiratory infections are most commonly transmitted through airborne droplets, droplet nuclei, or direct contact has not as yet been clearly established.

The techniques reported here represent a beginning toward the development of methods applicable to human studies aimed at the eventual answer to these questions. When applied in circumstances where environmental conditions are carefully controlled, and, especially when the isotopes are delivered in airborne suspensions comparable to naturally occurring aerosols, our knowledge should be improved. Now it should be possible to determine ranges of normal function in man of all ages, variations in normal function associated with environmental change, and association between such variations and airborne disease.

It is too early to say which of the three isotope techniques thus far explored is most useful or whether some other method may prove to be more effective. At the present time, it appears that the serial scans are most helpful in picturing the path of flow and, especially, in detecting areas of poor clearance. The double collimated detector seems most useful in quantitating speed of mucociliary flow between any two points. The single detector is the simplest mechanically.

These same techniques are also applicable to the study of areas of deposition of the isotope-labeled airborne particulates, as well as the determination of the eventual fate of those deposited.

At present, more questions regarding the upper respiratory tract and mucous membranes have been raised than answered. Among these are the following.

How much of the variability already noted is really "normal," and to what degree might the extremes of these "normal" variations be related to infection if they coincide with exposure? What pathological conditions in the upper respiratory tract increase susceptibility to infection? Are significant variations attributable to alterations in ciliary activity, respiratory tract mucus, or both? How are these functions affected by variations in environmental temperature or humidity, emotional stress, endocrine activity, pharmacological agents, etc.?

When these questions are clearly answered, research in the field of airborne disease may be more logically directed toward, or away from, the upper respiratory tract or the respiratory mucosa, or both.

SUMMARY AND CONCLUSIONS

Certain questions regarding the role of the upper respiratory tract in airborne disease remain unanswered. Among the most urgent are the following. What airborne materials are likely to be deposited upon the respiratory mucosa? What factors influence their removal from the air stream and their point of deposition? What is the fate of materials once deposited upon mucosal surfaces?

What factors influence their clearance, not only from the respiratory tract, but from the body?

Techniques are herein reported for the study of the respiratory air stream, and, through the external detection of radioisotopes, for the study of the deposition of airborne particles and their motion in the respiratory mucociliary stream. It is hoped that pursuit of these studies may cast some light on the transmission and pathogenesis of airborne disease.

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Measurement of Different Mechanisms for Elimination of Bacteria from the Lung

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INTRODUCTION

Several clinical and experimental studies have shown that the normal defense of the lung against bacteria might be affected by environmental agents, such as toxic gases (2), alcohol (7), and decreased temperature (3).

Although experimental studies of the disappearance rate of viable bacteria or of changes in mortality among bacteria-exposed animals are of great interest, a complete understanding of the recorded effects requires the study of the different elimination mechanisms separately. In turn, certain of the agents used to produce changes are able to affect one or more of these mechanisms.

This report presents a brief description of experimental methods used for the elucidation of the function of the different elimination mechanisms, and suggests an experimental set-up whereby the efficiency of the various mechanisms can be measured when the short-term elimination of bacteria from the lungs is being studied. In view of the bacteriological findings in chronic bronchitis (5), the present work has been performed with nonpathogenic bacteria, which were originally cultured from the mouths of the animals.

METHODS AND RESULTS

Guinea pigs were exposed for 10 min to a flow of radioactive bacteria in a monodisperse aerosol in a stainless-steel exposure chamber. The number of bacteria present at various sites in the lung was determined by use of bacteriological and autoradiographic (1) techniques. The number of bacteria at various times after the exposure was expressed as the percentage of the initial number remaining.

The number of viable bacteria in the whole lung was determined by use of a grinding technique similar to that described earlier (6). However, to ensure complete lysis of phagocytes, saponin was added to the grinding fluid in the present experiments. The number of viable bacteria was found to decrease fairly rapidly from these whole-lung preparations (Fig. 1). The results are generally in accordance with earlier reports. As the disappearance rate from the whole-lung preparations is a determinant of all the elimination mechanisms, the following experiments were performed to elucidate the individual functioning of the different mechanisms.

The airways were washed with sterile saline by use of a modification of the technique of LaBelle and Brieger (4). The lungs were flushed three times via the trachea with 10 ml of saline under aseptic conditions; then the number of bacteria in the fluid was determined. When *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, which all were present in the normal mouth flora, were used, it was found that the rate of decrease of viable bacteria in the fluid was more rapid than the decrease from whole-lung preparations (Fig. 1). If saponin was added to the wash-out fluid, a larger number of viable bacteria was found. If the autoradiographic technique was used, even more bacteria (*E. coli*) could be detected in the fluid (Fig. 2).

When the insides of isolated pieces of trachea, standardized with respect to length, were flushed with saline, the number of viable bacteria was found to be rather high during the first few hours after exposure but decreased later on. When saponin was added to the tracheal wash-out, a significant increase in the number of bacteria was only occasionally found (Table 1).

COMMENTS

The above findings suggest that, during the duration of these experiments, most of the *E. coli* that deposit on the mucus are carried upwards out of the lung without prior phagocytosis. The very rapid decrease in number of bacteria found

in the wash-out from the airways is apparently dependent upon the mucus transport. As viable bacteria and autoradiographically detectable bacteria could be expected to be removed by mucus at identical rates, the difference (Fig. 2) indicates that other mechanisms may be involved.

Although the wash-out fluid cannot be expected to remove all bacteria from the minor airways and alveoli, the presence of phagocytes in the airway wash-out fluid and the almost complete absence of phagocytes in the trachea wash-out fluid indicate that at least a certain number of the peripheral airways are subject to flushing with removal of free or loosely attached phagocytes. The difference in disappearance rates of viable bacteria and autoradiographically detect-

FIG. 1 *Decrease in viable bacteria (Escherichia coli) in whole lung (X) and in airway wash-out fluid (O)*
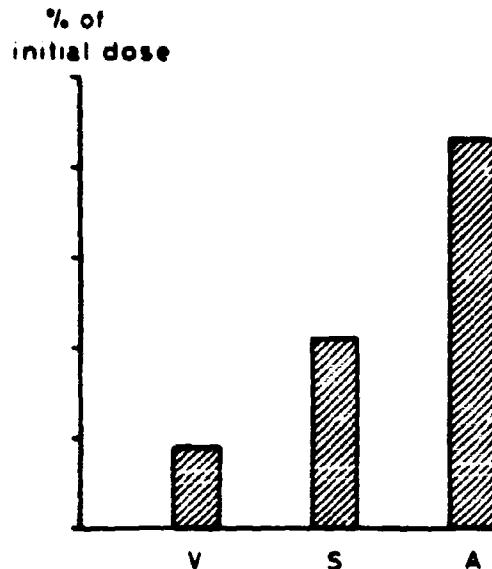
FIG. 2. *Number of Escherichia coli present in airway wash-out fluid 2 hr after exposure. V = viable; S = viable after saponin treatment; A = autoradiographically counted.*

TABLE 1. *Number of viable Escherichia coli cells in trachea wash-out fluid before and after saponin treatment*

				Time after exposure					
0 hr		1 hr		2 hr		3 hr		4 hr	
V*	S*	V	S	V	S	V	S	V	S
117	130	55	56	44	30	4	0	6	4
81	42	271	382	6	8	0	2	30	22
153	160	15	12	0	0	329	440	9	12
139	144	106	108	14	22	60	46	166	160
25	18	6	4	21	8	0	0	1	0
10	40	2	34	10	0	1	10	0	2
118	150	19	24	11	16	170	310	0	0
123	108	14	82	1	0	7	24	4	0
330	1,228	11	6	9	14	0	4	0	2
108	206	62	208	42	42	0	0	1	0

*V = viable E. coli cells before treatment; S = viable E. coli cells after saponin treatment.

able bacteria from the airway wash-out fluids could then be due to an uptake of bacteria by phagocytes, whereby the bacteria cannot be cultured but are autoradiographically detectable. This theory is supported by the observed difference in removal rates of viable bacteria with or without treatment of the wash-out fluid with saponin. The increased yield of bacteria when saponin is added to the fluid indicates that some bacteria initially retain their viability when within or adherent to the phagocytes.

The reduction in the number of bacteria in airway wash-out fluids is thus, apart from being a determinant of mucus trar port, also due to the removal of bacteria from the airways by *uptake in the phagocytes*.

The decrease in number of viable bacteria in the whole lung preparations is obviously the combined effect of mucus removal and of the bacteriocidal effect of the phagocytes.

Another elimination mechanism may be considered, namely, the removal of phagocytized bacteria out of the lung via an interstitial route. Cultures from blood and autoradiographic preparations from liver, kidney, and spleen did not reveal the presence of any bacteria, thus excluding the possibility that direct elimination via the blood plays a major role in the present experiments and with the types of bacteria studied here.

To be able to test the elimination mechanism discussed here, one would have to use two different organisms, one of which i subject to only one of the elimination mechanisms. Spores seem to be suitable for this purpose.

The efficiency of the mucus removal, of the phagocytic removal from the airways, and of the bactericidal effect of the phagocytes in the lung can thus be tested with the following model

The animals are exposed to an aerosol containing radioactive spores and nonradioactive bacteria, e.g., *E. coli*. The reduction in the number of spores in whole lung grinding will then give an estimate of the capacity of the mucus removal. The reduction in the number of viable bacteria in wash-out fluids from the airways will give an estimate of the capacity of the phagocytic and mucus removal, and the reduction of viable bacteria in whole-lung grindings

will provide an index of the bactericidal activity of the phagocytes in the lung and of mucus removal. Effects on any of the above functions can then be evaluated, provided that the three functions are tested simultaneously and that the effects are expressed as deviations from the normal.

Preliminary results from experiments performed with the above model indicate that the mucus removal during the first 2 hr after exposure accounts for about 15% of the total removal under the experimental conditions used. This rate agrees with results from experiments where the elimination of monodisperse plastic aerosols of various size ranges has been followed (Holma, *personal communication*). Experiments are in progress wherein the possible effects on any of the three elimination mechanisms discussed above of continuous exposure to low doses of SO_2 and to dust are being evaluated.

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Effect of Route of Inoculation on Experimental Respiratory Viral Disease in Volunteers and Evidence for Airborne Transmission

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INTRODUCTION

Initiation of respiratory viral infection, with some possible exceptions, appears to depend upon deposition of infectious virus at some point on the respiratory tract. There appear to be two possible mechanisms of transmission, contact or airborne. The former term is meant to refer to transfer of virus by physical contact between an infected and a susceptible subject, or indirectly through personal articles or fomites. Transmission by this route would result in deposition of virus predominantly in the nasopharynx.

Airborne transmission is intended to mean transfer of infection by means of small-particle aerosols (11, 16). These particles are the evaporated residues of infected respiratory secretions which are of such small size (mostly less than 5 μ in diameter) that they will remain airborne for long periods of time. As a function of their small size, such droplets, when inhaled,

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deposit predominantly in the lower respiratory tract. Particles between 5 and 15 μ to 20 μ in diameter represent an intermediate stage, and most particles in this size range will be trapped in the nose, although some will penetrate to below the larynx. (Lower respiratory tract will refer to that portion of the respiratory tract below the larynx.) Still larger particles may be produced by coughing and sneezing, etc., but since, because of their large size, they do not produce stable aerosols, transmission will ordinarily occur only by direct impaction on the nasopharynx of persons in the immediate vicinity of an infected case. Such transmission would be difficult to distinguish from that resulting from contact, and is best considered under this category.

This report will describe studies of the transmission of respiratory viral diseases which were a joint undertaking of the U.S. Army Biological Laboratories, Fort Detrick, Md., and the Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

The first part of the report will describe an investigation of the infectiousness of respiratory viruses given by methods which attempt to simulate natural contact and airborne transmission, namely, nasal drops and aerosols containing virus. Coxsackievirus A type 21, a strain of rhinovirus, and adenovirus type 4 were used in these studies.

The second part will describe recovery of virus from natural aerosols produced by coughing and sneezing and from air of rooms contaminated by such discharges. In addition, preliminary results of an experimental attempt to transmit respiratory viral infection in volunteers by the airborne route will be presented.

MATERIALS AND METHODS

Volunteers

Subjects were healthy adult male inmates from several federal correctional institutions and were selected on the basis of serum antibody determinations, willingness to participate, and demonstration of good health. For studies performed at the Clinical Center, National Institutes of Health, volunteers were isolated two or three to a room for 3 to 4 days prior to inoculation and 10 to 14 days after inoculation. Examinations were performed daily by physicians having no knowledge of which of several respiratory agents was administered to a particular volunteer.

An experimental transmission experiment was performed at the Federal Prison Camp, Eglin Air Force Base. Fla. Volunteers were housed in a converted barracks building, and were evaluated before inoculation and twice daily after inoculation by physicians who knew which volunteer was inoculated and which was an exposed susceptible. Complete separation of the two groups, as described in the text, was carefully maintained; however, only partial separation from the remaining camp population was maintained.

Inocula

Virus strains used in these studies were obtained from Marines with acute respiratory disease at Parris Island, S.C., or Camp Lejeune, N.C. (through the courtesy of K. M. Johnson, H. H. Bloom, and R. M. Chanock). Each inoculum had been passaged once or twice (see Results) in either human embryonic kidney (HEK) or human embryonic fibroblast (HEF, strain WI-26) tissue cultures (17). The harvests in each case were frozen and thawed, pooled, centrifuged at $1,000 \times g$ for 20 min, and filtered through 800-mμ membrane filters (Millipore).

The filtrates were stored at -60 C until used. Each inoculum was safety-tested for adventitious agents in a manner previously described (19). In addition to the above described procedures, the coxsackievirus A type 21 strain 48654 HEF$_2$ was submitted to vacuum concentration and trifluortrichlorethane (Gelman) treatment. Further details of these procedures have been described (6, 8, 9).

Inoculation Procedures

Volunteers received aerosol inoculation by means of a molded rubber face mask attached to a cylindrical chamber containing a continuous flow of aerosol generated by a Collison atomizer. Virus was approximately 10 sec old at the time of inoculation. This equipment and other necessary auxiliary components were contained in a mobile truck and semitrailer and have been previously described (15). Each man inhaled 10 liters ($\pm 5\%$) through the nose, and exhaled by mouth into a discharge bag. Each inoculation required 30 to 60 sec and usually followed a training period on a previous day with use of the same equipment. The size of particles in the aerosol ranged from 0.2 to 3.0 μ in diameter. Particles 1 to 2 μ in diameter comprised 54% of the total particle volume and contained 68% of recoverable virus. Further details of the aerosol will be described in a subsequent report in this symposium (14). Aerosol inoculations with particles 15 μ in diameter were performed with the same equipment, except that the vibrating reed method of Wolf was used to generate the aerosol (25). Volunteer doses for both aerosols were calculated from virus assays in simultaneously collected Shipe impinger samples of the aerosol.

Nasopharyngeal inoculations were performed by the instillation of 0.25 ml of virus inoculum into each nostril of the volunteer while he was prone. This inoculation was accompanied by a sensation of liquid in the nose but not by a desire to expectorate or swallow. In addition, some volunteers received 0.5 ml of inoculum into each nostril as well as 0.5 ml sprayed into each nostril by a no. 127 DeVilbiss (12) hand atomizer. Studies on the aerosol produced by this atomizer have shown that 99.95% of the inoculum is contained in particles greater than 5 μ in diameter and most would be deposited in the nasopharynx (unpublished data).

Collection of Cough, Sneeze, Talking, and Room Air Samples

Particles produced in selected expiratory events were collected for size analyses and virus

assay. In addition, room air samples were collected in a large-volume air sampler. Description and analysis of the methods used will also be described in a subsequent report in this symposium (14).

Virus Isolations and Identification Procedures

Specimens obtained varied with the virus being studied but included nose, throat, and anal swabs, nasal washes, and expectoration specimens. Specimens were collected prior to and subsequent to inoculation. Expectoration specimens were stored in that form until tested. Nasal washes were performed with 10 ml of Veal Infusion Broth (Difco) containing 0.5% bovine albumin with antibiotics; swabs were agitated in 2 ml of this medium and then discarded. All specimens were stored at −20 C until tested. Testing for virus was performed by inoculating 0.4 ml of specimen fluid into one HEK and HEF tissue culture tube that contained 1.5 ml of equal parts of medium 199 and Eagle's MEM, 2% inactivated calf or chicken serum, and antibiotics. The cultures were incubated in a roller drum turning at 12 rev/min at 33 to 34 C and were observed for cytopathic effect (CPE). This observation period was 14 days for coxsackievirus A type 21 and rhinovirus NIH 1734, but 60 days for adenovirus type 4. All the latter studies were performed in HEK cultures. Tissue culture fluid and cells were harvested when CPE involved 75 to 100% of the cell sheet. For coxsackievirus A type 21 and adenovirus type 4, the first and last isolates, as well as intervening isolates, when indicated, were identified by hemagglutination-inhibition (HI) with 20 antibody units of specific hyperimmune guinea pig serum or rabbit serum. HEF cultures were used for identification of comparable specimens of rhinovirus NIH 1734 by neutralization of 32 to 100 TCID₅₀ of virus with specific hyperimmune guinea pig serum. Further details of these procedures have been reported (6, 8, 9).

Serological Tests

Serial fourfold dilutions of inactivated serum were tested for neutralizing antibody for each virus by mixing equal volumes of the serum dilution with a test dose of virus, incubating at room temperature, inoculating each of two tissue culture tubes with 0.2 ml of the mixture, and observing thereafter for CPE.

All neutralizing antibody titers, calculated by the method of Karber, are expressed as the initial dilution of serum completely inhibiting CPE of 32 to 100 $TCID_{50}$ of coxsackievirus A type 21 and adenovirus type 4, but 10 to 16 $TCID_{50}$ of rhino-

virus NIH 1734. Further details of the procedures have been reported (6, 8, 9, 13).

RESULTS

Response to Inoculation with Aerosol and Nasal Drops

Coxsackievirus A type 21: 50% human infectious doses (HID_{50}). Volunteers free of detectable antibody were inoculated with a range of doses of coxsackievirus A type 21 by small-particle aerosol (diameter of particles, 0.3 to 2.5 μ), large-particle aerosol (diameter of particles, 15 μ), and nasal drops (0.25 ml in each nostril). An example of the type of response obtained is shown in Table 1. Twenty-eight volunteers received strain 49889 HEK_1 in a small-particle aerosol, and 18 became infected. The doses, number of volunteers who received each dose, and the number who became infected, as determined by virus isolation and antibody rise, are shown. Based on these findings, the HID_{50} for this inoculum administered in this way corresponds to 28 $TCID_{50}$ (Spearman-Karber method; 13). Only two of the infected volunteers failed to develop illness, indicating that the 50% infectious dose and 50% illness dose are nearly the same.

In this experiment, three volunteers developed unexplained mild cases of rhinitis. Experience with over 300 volunteer inoculations indicates that such an illness is recorded in about 15% of uninfected individuals. The phenomenon occurs even though virus is inactivated with specific hyperimmune serum, in men with all levels of serum antibody, and irrespective of virus type or materials and methods used for inoculum preparation (8). Attempts to isolate a causative agent in HEK, HEF, and rhesus monkey kidney tissue cultures have been unsuccessful.

The HID_{50} for strain 49889 HEK_1 and another inoculum (strain 48654 HEF_2) of coxsackievirus

TABLE 1. Response of antibody-free volunteers inoculated with 0.3 to 2.5-μ particle aerosol of coxsackievirus A type 21 (strain 49889 HEK_1)*

Inhaled dose (TCID₅₀)	No. of volunteers	No. infected	No. ill
832	1	1	1
676	3	3	2
316	3	3	3
83	2	2	2
71	5	5	4
47	4	3	3
18	4	1	2ᵇ
6	6	0	2ᵇ

* HID_{50} = 28 $TCID_{50}$.
ᵇ Three cases of afebrile URI without infection.

TAB·E 2. HID_{50} for coxsuckievirus A type 21

Inoculum	Inoculation method	No. of volunteers	No. infected	HID50	95% Confidence limits
Strain 49889 HEK₁*	Aerosol, 0.3 to 2.5-μ particles	28	18	28 TCID₅₀	15–49
Strain 48654 HEF₂*	Aerosol, 0.3 to 2.5-μ particles	14	8	34 TCID₅₀	22–52
	Aerosol, 15-μ particles	29	12	32 TCID₅₀	13–78
	Nose drops	14	7	6 TCID₅₀	3–13

* One passage in human embryonic kidney tissue cultures.
* Two passages in human embryonic fibroblast tissue cultures.

TABLE 3. Clinical response of antibody-free volunteers to coxsuckievirus A type 21

Inoculum	Inoculation method	No. of volunteers	No infected	No. ill	Predominant illness		
					Afebrile URI*	Febrile URI	Febrile LRI*
Strain 49889 HEK₁*	Aerosol, 0.3 to 2.5-μ particles	28	18	16	1	3	12
	Coarse spray and nose drops	13	13	8	2	6	
Strain 48654 HEF₂*	Aerosol, 0.3 to 2.5-μ particles	14	8	8	1	7	
	Aerosol, 15-μ particles	29	12	11	1	8	2
	Nose drops	14	7	5	2	3	

* Upper respiratory tract illness.
* Lower respiratory tract illness.
* One passage in human embryonic kidney tissue cultures.
* Two passages in human embryonic fibroblast tissue cultures.

A type 21 administered by each of the described methods in shown in Table 2. As can be seen, the HID_{50} is virtually identical for the three aerosol titrations; however, for virus administered by nasal drops, it is about fivefold less. Natural virus (virus recovered from naturally infected individuals, but not cultivated in vitro) administered by small-particle aerosol (not shown) produced infection in one of two volunteers at a dose of 28 TCID₅₀, and in none of six who received 7 TCID₅₀, suggesting a similar degree of infectivity (8).

The HID_{50} for each aerosol inoculum is based on inhaled virus. Available information indicates that only 50 to 75% of particles of the size range in the small-particle aerosol would be retained and that the majority of these would deposit in the lower respiratory tract (11, 16). This indicates that the true HID_{50} for the inocula administered in this way is appreciably less than that indicated in Table 2. All of the nasal drop inoculation was retained, and therefore the HID_{50} for this method corresponds to the HID_{50} given in the table. Since virtually all 15-μ particles

would be retained, and the majority would be trapped in the nose, one would expect the HID_{50} by this route of inoculation to be similar to that obtained by nasal drops. No explanation is presently available for the observed difference.

The clinical responses that correspond to the strains and inoculation methods in Table 2 are shown in Table 3. In addition, the responses to 3,000 TCID₅₀ of strain 49889 HEK₁ administered to the nasopharynx by coarse spray and drops are included (22). The frequencies of occurrence of illness in each of the five categories were not significantly different. As can be seen, the predominant clinical response to strain 49889 inoculated by small-particle aerosol was febrile lower respiratory tract illness. All 12 volunteers with this response were clinically diagnosed as having acute tracheobronchitis. The pertinent data obtained on a volunteer from a more recent experiment, but typical of the syndrome, are shown in Fig. 1. Characteristic of this syndrome was the occurrence of pain in the neck (tracheal) and chest, the latter usually being substernal. Cough, often paroxysmal, was usually non-
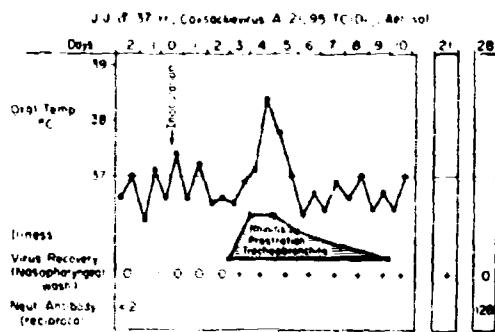
FIG. 1. *Case report of an antibody-free volunteer inoculated with coxsackievirus A type 21 by small-particle aerosol.*

productive, although auscultation of the chest occasionally revealed scattered rhonchi, and, in two cases, there was X-ray evidence of pneumonia. These lower respiratory tract symptoms were accompanied by malaise, myalgias, chilly sensations, sweats, headache, and anorexia. Illness was not limited to the lower respiratory tract, however, since 9 of the 12 volunteers with tracheobronchitis also had upper respiratory tract illness that was characterized by rhinorrhea and nasal obstruction. Four of the remaining six infected volunteers had upper respiratory tract illness only, and the other two had infection without apparent illness.

In contrast to the small-particle aerosol response, 8 of 13 volunteers who received nasopharyngeal inoculation developed upper respiratory tract (nasopharyngeal) illness only. The fact that virus was deposited in the nasopharynx in this case and predominantly in the lower respiratory tract in the former suggested that virus deposition site accounted for this difference and that it might be the factor that determines the clinical response. However, when strain 48654 was administered by small-particle aerosol, the lower respiratory tract illness, which was characteristic of strain 49889 given in this way, was not seen. The predominant clinical response to strain 48654 in a small-particle aerosol was febrile upper respiratory tract illness (Table 3). Thus, virus deposition site and inoculum differences both appeared as important factors in determining the type of clinical response.

Febrile upper respiratory tract illness was also the predominant clinical response for strain 48654 administered by 15-μ particle aerosol and by nasal drops (Table 3). Not shown are clinical responses to natural virus and to still another strain of virus administered by small-particle

aerosol (8, 20) For these inocula, febrile upper respiratory tract illness also predominated. This combined experience suggests that virus deposition site may be an important factor in determining the type of clinical response that occurs. However, for coxsackievirus A type 21, most strains appear to lack the capability of producing lower respiratory tract illness when presented such an opportunity by virus deposition at this site.

In all other aspects, the clinical responses were similar for each inoculum and inoculation method. The incubation period was 2 to 5 days, illness usually lasted 2 to 3 days, fever rarely exceeded 38.5 C, and fever usually persisted less than 1 day.

The effect of pre-existing serum neutralizing antibody on the responses following inoculation of volunteers with coxsackievirus A type 21 (strain 49889) has not been completely evaluated, but the data available are shown in Table 4. As can be seen, all individuals with intermediate titers of antibody were infected after nasopharyngeal inoculation, but infection occurred in only 5 out of 11 with high titers. A similar suggestion of reduction in infection also occurred in the small-particle aerosol groups.

Rhinovirus NIH 1734: HID_{50}. Volunteers free of detectable antibody to this virus were inoculated with a range of doses of rhinovirus NIH 1734 by small-particle aerosol and by nasal drops. The HID_{50} for each inoculation method is shown in Table 5. Nasal drop doses of 1 $TCID_{50}$ and less were extrapolated values based on dilutions of a pool with known virus concentration, and aerosol doses of 2 and less were extrapolated from measured concentrations of virus in aerosols produced, during the inoculation period, by higher concentrations of virus. Repeated tests of several dilutions of virus run in sequence have been shown to produce proportionate changes in aerosol virus concentration. Assays for virus were performed in HEF (WI-38) tissue cultures, in a

TABLE 4. *Response of volunteers with pre-existing antibody to inoculation with coxsackievirus A type 21*

Level of antibody	Nasopharyngeal inoculation			Aerosol, 0.3 to 2.5 μ particles		
	No. of volunteers	No. infected	No. ill	No. of volunteers	No. infected	No. ill
Intermediate (1:4-1:128)	6	6	4	5	3	2
High (1:256 or greater)	11	5	0	3	0	3*

* Each illness was mild rhinitis.

TABLE 5. HID₅₀ for rhinovirus NIH 1734

Inoculation method	No. of volunteers	No. infected	HID₅₀	95% Confidence limits
Nasal drops	17	11	0.032 TCID₅₀	0[a]
Aerosol, 0.3 to 2.5 μ particles	26	20	0.68 TCID₅₀	0.2–2.0

[a] Indicates no intermediate response between 100 and 0% infection.

TABLE 6. Clinical response of volunteers to inoculation with rhinovirus NIH 1734

Inoculation method	No. of infected volunteers	No. ill	Illness		
			URI	URI, LRI[a]	LRI
Coarse spray and nose drops	48	43	41	2	0
Aerosol, 0.3 to 2.5 μ particles	41	33	23	5	5

[a] Upper and lower respiratory tract illness.

manner described previously (6). Other types of tissue culture [HEK and HEF (WI-26)] and tissue culture assay [HEF (WI-38) plaque assay] were tested and found to be equal to or less sensitive than the cultures and methods used.

As can be seen in Table 5, the HID₅₀ for both inoculation methods was below the practical limits of detection. Failure to infect all volunteers with small-particle aerosol inoculation first occurred at an inhaled dose of 2 TCID₅₀, and none of three who inhaled 0.06 TCID₅₀ became infected. The HID₅₀ for this inoculation method was 0.68 TCID₅₀ (Spearman-Karber; 13). In contrast, all volunteers who received 0.1 TCID₅₀ by nasal drops became infected, although none became infected at two lower doses The HID₅₀ for this method corresponded to 0.032 TCID₅₀. These results indicate an approximately 20-fold disparity between infectivity for the virus given by the two methods. The disparity could be accounted for by assuming that the 10 to 20% of small-particle aerosol particles that deposit in the nasopharynx are responsible for all infection in volunteers inoculated in this way. However, the fact that this is not the case is suggested by the occurrence of lower respiratory tract illness in some of these volunteers. In any event, the data suggest that the nasal mucosa is somewhat more susceptible to rhinovirus NIH 1734 than is the lower respiratory tract. Although the difference was less for coxsackievirus A type 21, it was similar in direction.

The clinical responses of all volunteers who have received either nasal or small-particle aerosol inoculation with rhinovirus NIH 1734 are shown in Table 6. As can be seen, the characteristic response to either method of inoculation is an upper respiratory tract illness which in all respects is a common cold. The pertinent data obtained from one of the volunteers inoculated by nasal drops are shown in Fig. 2. His response consisted of a common cold syndrome characterized by nasal obstruction and discharge, and was accompanied by throat irritation and systemic symptoms. The extent of the rhinorrhea

is shown in the figure. Fever was absent in this volunteer and occurred in less than 10% of the volunteers, regardless of method of inoculation.

As can be seen in Table 6, lower respiratory tract illness (acute tracheobronchitis) was predominant in five volunteers who received small-particle aerosol inoculation, and diffuse respiratory tract disease without a predominant localization was seen in five others. Predominant lower respiratory tract illness was not seen in men inoculated by nasal drops, although two volunteers exhibited a combination of upper and lower respiratory tract illness. These findings suggest that aerosol inoculation may produce lower respiratory tract involvement, but the characteristic response to infection produced by either method is an upper respiratory tract illness.

The incubation period of the illnesses produced by both inoculation methods was 2 to 4 days, the illness usually lasted 2 to 3 days, and fever, when it occurred, was usually 1 day in duration.

The effect of pre-existing serum neutralizing antibody on responses to inoculation with rhinovirus NIH 1734 is shown in Table 7. As can be seen, no significant reduction in frequency of infection occurred unless high levels of serum antibody were present. This reduction in frequency of infections occurred for both methods of inoculation and was accompanied by a similar reduction in illnesses. [Data are grouped for convenience. Individual values were tested in Spearman's rank correlation or Yates mean score tests (13). Reduction in infection and illness with increasing serum antibody was statistically significant (P < 0.05) for both inoculation methods.]

Adenovirus type 4: HID₅₀. Nine volunteers free of detectable antibody to adenovirus type 4 received small doses of this virus by small-particle aerosol. Six volunteers received the virus by 15 μ particle aerosol. The results of these

RHINOVIRUS COMMON COLD - W.W.A., 38 Yr.♂ VOLUNTEER

Day After Inoculation	-2	-1	0	1	2	3	4	5	6	7	8	9	10	13	21

DAILY NASAL SECRETION (GMS)

Illness — Rhinitis, Malaise, Throat Irritation

NIH 1734

Nasopharynx Neut. Antib.
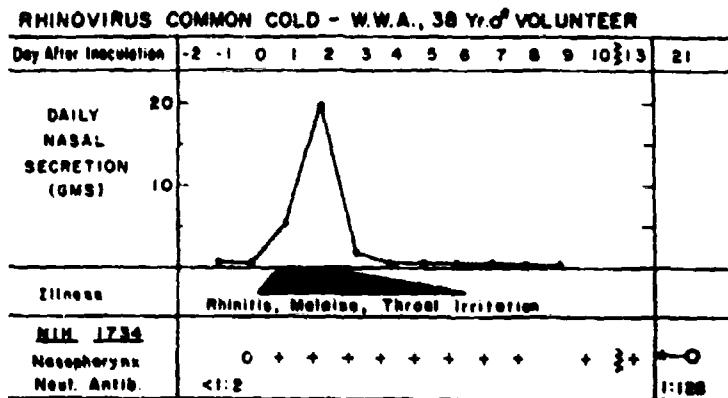
Fig. 2. *Case report of an antibody-free volunteer inoculated with rhinovirus NIH 1734 by coarse spray and nasal drops. (Reproduced with the permission of the Journal of Clinical Investigation.)*

studies are shown in Table 8. As can be seen, all volunteers who received doses of 11 and 5 $TCID_{50}$ by small-particle aerosol became infected, but only one out of three became infected at a dose of 1 $TCID_{50}$. Other volunteers were inoculated in this way, and, although the data are incomplete, the studies indicate that the HID_{50} for small-particle aerosol inoculation is about 1 $TCID_{50}$. It should be stated that these virus assays were performed in HEK tissue cultures, the most sensitive tissue available for adenovirus, and the cultures were observed for 60 days for CPE with subpassage as needed. This time period was shown to provide maximal detection of adenovirus (9).

Only one dose level of adenovirus type 4 has been administered by 15-μ particle aerosol, and this was 1,000 $TCID_{50}$. All six volunteers who received this dose became infected. Preliminary results on inoculation of volunteers by nasal drops indicate that the HID_{50} by this method is about 20 $TCID_{50}$. This combined experience with adenovirus type 4 suggests that a greater dose of this virus is required to initiate infection in the nasopharynx than in the lower respiratory tract.

Also shown in Table 8 are the clinical responses seen in the volunteers inoculated by aerosol. As can be seen, all volunteers infected by means of small-particle aerosol inoculation became ill, and the illness was usually febrile. Three volunteers had predominantly upper respiratory tract illness, and, in three others, illness was predominantly in the lower respiratory tract. The latter included one instance of mild pneumonia. Only three of the six volunteers infected by 15-μ particle aerosol inoculation became ill, two with febrile upper respiratory tract illness and one with pneumonia. The incubation period for these

TABLE 7. *Response of volunteers with pre-existing antibody to inoculation with rhinovirus NIH 1734*

Level of antibody	Nasopharyngeal inoculation			Aerosol, 0.3 to 2.5 μ particles		
	No. of volunteers	No. infected	No. ill	No. of volunteers	No. infected	No. ill
Low (1:2-1:8)	1	1	2	5	4	4
Intermediate (1:16-1:64)	9	8	7	8	6	4
High (1:128 or greater)	13	8	4	4	1	1

illnesses varied between 6 and 13 days, duration of illness varied between 2 and 10 days, and fever between 1 and 8 days. In addition, the severity of illness, as manifested in respiratory tract involvement and constitutional symptoms, also was quite variable. Upper respiratory tract findings occurred in all men in the 15-μ particle aerosol group, whereas this finding was variable in the small-particle aerosol group. The pertinent data obtained on one of the volunteers who exhibited the syndrome described as acute respiratory disease (ARD) of military recruits are shown in Fig. 3. Bacteriological cultures were negative for pathogens, and spontaneous recovery occurred without antibiotic therapy.

It is notable that the syndromes of febrile respiratory tract illness that occurred after aerosol inoculation resemble the naturally occurring type 4 adenovirus diseases of military recruits (5, 7, 21). Previous studies by others, in which volunteers were inoculated in the nasopharynx, usually resulted in asymptomatic infection or mild afebrile upper respiratory illness (1). Inocula-

TABLE 8. *Response of antibody-free volunteers to adenovirus type 4*

Inoculation method	Dose[a]	No. of volunteers	No. infected	No. ill	Illness		
					Afebrile URI[b]	Febrile URI	Febrile LRI[c]
Aerosol, 0.3-2.5-μ particles	11	3	3	3	1	1	1
	5	3	3	3		1	2
	1	3	1	1		1	
Aerosol, 15-μ particles	1,000	6	6	3		2	1

[a] Expressed as TCID₅₀.
[b] Upper respiratory tract illness.
[c] Lower respiratory tract illness.
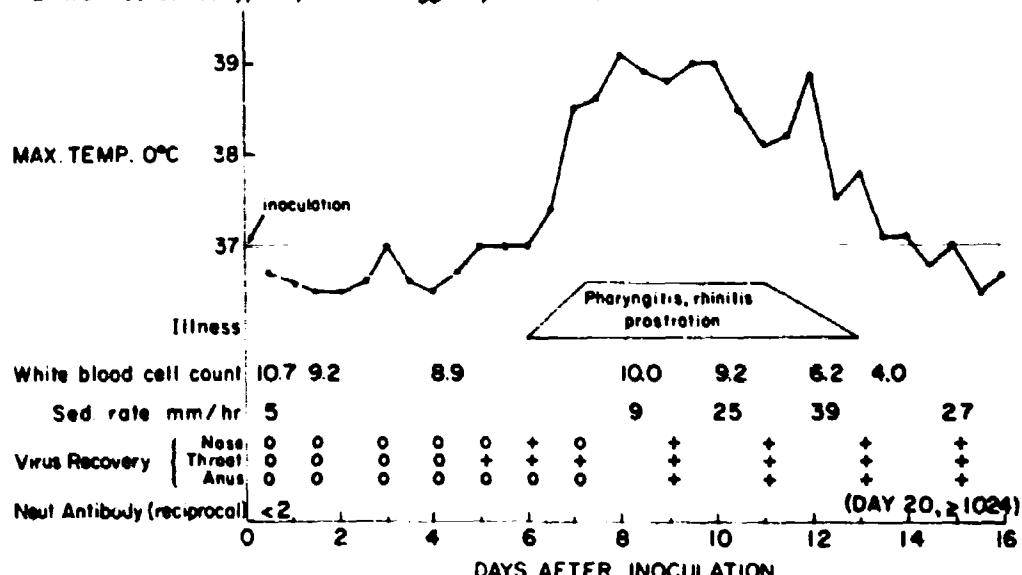
FIG. 3. *Case report of an antibody-free volunteer inoculated with adenovirus type 4 by 15-μ particle aerosol White blood cell counts are times 10³ per cm. (Reproduced with the permission of the American Review of Respiratory Diseases.)*

tions into the conjunctival sac resulted in occurrence of conjunctivitis only or pharyngoconjunctival fever, illnesses which rarely occur naturally in type 4 infection, and which were not seen in the present studies (1). These findings suggest that the unique feature of the present inoculations, deposition of virus in the lower respiratory tract, was the major factor accounting for the recruit type illnesses. This is supported by the fact that small doses of virus given by small-particle aerosol produced illness in all volunteers infected by this method of inoculation,

whereas the large dose given by 15-μ particle aerosol caused illness in only three of six infected men. Evidence indicates that most of the 15-μ particles were deposited in the upper respiratory tract, but the possibility exists of deposition in the lower respiratory tract either by direct inhalation or as a result of particle fragmentation (16). It is suggested that in three men this occurred and caused febrile illness.

Three volunteers with pre-existing antibody titers of 1:32 to 1:64 received 6 TCID₅₀ by small-particle aerosol, and none became infected or ill.

Inoculations of volunteers with pre-existing antibody into the nasopharynx or conjunctival sac by others have also demonstrated the protective effect of serum antibody (1).

Evidence for Airborne Transmission

Detection of virus in particles produced by coughing, sneezing, and normal expiration. By use of methods (14) for recovery of virus from particles produced by coughs and sneezes, virus titration was carried out on 61 cough collections and 58 sneeze collections from volunteers infected with coxsackievirus A type 21 (Table 9). The collection method involved coughing or sneezing into a collapsed weather balloon through a tight-fitting face mask. The air in the balloon was evacuated through a Shipe impinger to remove airborne particles, and material impacted on the wall of the balloon was collected by rinsing with sterile tissue culture fluid. When the results of both samples were combined, 39% of cough specimens and 50% of sneeze specimens were positive for virus. Thirty per cent of air samples were positive for both events, and the mean quantity present was 30 TCID$_{50}$ and 60 TCID$_{50}$ for cough and sneeze samples, respectively. This close similarity in results is of interest in view of the approximately 20-fold greater number of particles and particle volumes produced by sneezing (14). This finding suggests that the concentration of virus in secretions released in small particles produced by coughing is greater than that produced by sneezing.

Analysis of balloon wall samples revealed a disparity between the two events. Wall samples of sneezes were more frequently positive than the wall samples of coughs, but the mean quantity present was only twofold greater. However, the mean quantity of virus present in the wall samples from sneezes does not include four sneezes in which gross contamination with large quantities

of nasal secretion occurred. The wall samples of these sneezes contained 30,000 to 500,000 TCID$_{50}$ of virus. The reasons for the disparity in frequency of detection of virus on the balloon wall for the two events are not known at the present time, since studies have revealed similar particle size distributions for both events (12, 14).

Breathing samples were tested by collecting the entire amount of expired air in Shipe impingers through a closed system for 30-min periods. This testing constituted sampling of air expired for 2 hr per day from four infected volunteers during the period that included occurrence of illness and maximal virus shedding. A volume equivalent to 12 hr of expired air was tested in this way, and all samples were negative for virus.

A number of factors were evaluated to determine the cause for virus release in the process of coughing and sneezing. These evaluations suggested that the presence of nasal obstruction and discharge was the most important determinant for release of virus when infected persons sneeze [with nasal obstruction and discharge, 19 of 24 sneeze samples were positive; without nasal obstruction and discharge, 11 of 34 samples were positive ($P < 0.001$)]. In contrast, positive cough specimens bore a relationship only to the quantity of virus present in respiratory secretions, and this relationship occurred for air samples only [combined nasal and oral secretions, Yates mean score, test, $P = 0.05$ (13)]. Since cough particles would presumably be derived from pharyngeal and lower respiratory secretions, it is suggested that the concentration of virus in these secretions varied proportionately with the secretions tested.

Virus in room air. The contribution to room air contamination by coughing, sneezing, and possibly by other expiratory phenomena of man involves frequency and occurrence of the phenomenon, inactivation of virus, and physical loss of aerosol particles, in addition to quantity of virus released. The significance of these factors in determining environmental contamination was tested by collecting particles present in the air of rooms occupied by volunteers infected with coxsackievirus A type 21 and then assaying the collections for virus.

The large-volume air sampler was used to collect particles from approximately 70% of room air after a period of 2 hr with no ventilation. Shown in Table 10 are the results of testing 30 such samples collected during the acute phase of illness and maximal virus shedding. Of the 30 samples, 14 were positive, and, as can be seen, the frequency of positive samples increased with increasing quantity of virus present in respiratory

TABLE 9. *Virus recovery from particles in coughs and sneezes produced by volunteers infected with coxsackievirus A type 21*

Phenomenon	No. tested	Percent positive	Source	Percent positive	Mean quantity (TCID$_{50}$)
Sneeze	58	52	Air[a]	30	60
			Wall[b]	45	100
Cough	61	39	Air[a]	30	30
			Wall[b]	20	50

[a] Assay of Shipe impinger collection of particles suspended in air in balloon.

[b] Assay of 10-ml liquid rinse of balloon wall.

TABLE 10. *Relation of virus quantity in respiratory secretions to virus in room air samples*

Mean (3 vol.) virus quantity in secretions	Air sample		
	No. of tests	No. positive	Mean virus quantity
10-30[a]	5	1	5[a]
30-100	11	2	160
100-300	5	4	230
300-1,000	6	4	90
1,000->1,000	3	3	300

[a] Expressed as $TCID_{50}$ per milliliter of secretion.
[b] Expressed as $TCID_{50}$.

secretions [Smirnoff test, $P < 0.01$ (13)]. The mean virus quantity in positive samples is shown in the last column and was sufficiently variable so that no quantitative relationship to virus in respiratory secretions was detected, although it is of interest that the largest quantity present in room air, 28,000 $TCID_{50}$, was in the room with the highest virus concentration in secretions.

Since both positive cough and room air samples were related to quantity of virus in respiratory secretions of infected volunteers, it was suggested that coughing was responsible for contamination of room air with virus. When the results were analyzed by room, it was found that the presence of virus in cough air samples from volunteers occupying a room was significantly related to the recovery of virus from the air of that same room on the same day. [Positive room air samples, 10 of 11 rooms with positive cough air samples; negative room air samples, 2 of 7 rooms with positive cough air sample ($P = 0.03$)]. This further suggested that cough is the important intermediary between virus in secretions and virus in room air. No such relationship was detected for sneezing. These findings are not surprising, since cough as a symptom was recorded as being frequently present in these same volunteers at this time, whereas sneezing was not.

Preliminary report on a transmission experiment. An experiment designed to test whether the occurrence of air contamination with virus is sufficient to produce airborne transmission has been performed (*unpublished data*). Nineteen placebo-inoculated volunteers were exposed to air surrounding infected volunteers by housing the two groups in a converted barracks and separating them with a double wire barrier. Even distribution of air on both sides of the test building was accomplished by means of large floor fans and was proved by generating an aerosol containing a fluorescein dye on one side and then collecting and analyzing air samples from different locations throughout the building. Coxsackievirus A type 21 infection was produced in 10 volunteers with aerosol inoculation, and all exposed individuals became infected with this virus during the course of the study. A specific separation of results in terms of contact and airborne-acquired infection is not completed, but it is possible to state that airborne transmission unquestionably occurred.

DISCUSSION

The theory that respiratory viruses are transmitted by the airborne route has been popular in the past, primarily because it seemed reasonable to assume that coughing and sneezing, common symptoms of viral respiratory disease, produce aerosols that would accomplish such transmission. Despite this assumption, proof that man produces aerosols that contain virus and that sufficient viral contamination of air occurs to result in this type of transmission, both essential requirements for airborne transmission, has not been obtained (24). The results presented in this report provide this important information. It was shown that individuals infected with respiratory viruses, in this case coxsackievirus A type 21, produce airborne virus in quantities sufficient to infect susceptible individuals. The capacity to produce viral aerosols was tested for three expiratory events. Breathing samples were uniformly negative for virus, whereas cough and sneeze samples were frequently positive. Thus, whereas in man the former event is probably in significant in producing transmission of the respiratory viruses, it seems likely that it is important in the mouse-influenza system of Shulman and Kilbourne (23) in which airborne transmission has also been conclusively demonstrated. For man, coughing and sneezing appear to be the significant events for producing viral aerosols.

Studies in which virus released by coughing and sneezing was collected in a balloon and separated into an air phase and a wall phase provided quantitative results that correspond roughly to virus involved in airborne transmission and contact transmission, respectively. Virus was recovered more often from the air sample from coughs than from the wall samples, although the wall samples of sneezes were more commonly positive than the air samples. This would suggest that sneezing may be of some significance for that form of transmission involving direct impaction of large particles in the nasopharynx, whereas cough contributes primarily to small particle

aerosol transmission. Despite the differences in frequency of recovery, the difference in mean quantity of virus in each phase was small and quite similar for each event. These findings are in contrast to the findings of Buckland et al. (5), in which the vast majority of virus released in sneezing was found on the sides of a large sampling bag. However, the different collection methods involved may account for this disparity.

Despite a larger number of particles in sneezes than in coughs, the quantity of virus expelled in the two events was remarkably similar, suggesting that, in these volunteers, the concentration of virus in secretions atomized in coughing was relatively greater than that in secretions atomized in sneezing (12, 14). Inoculation of these volunteers was performed by small-particle aerosol, and, although lower respiratory tract secretions were not quantitated, virus is known to have been deposited at this site and probably induced infection there. Thus, it is possible that the method by which infection was induced may have contributed to the virus recovery results from coughing.

The fact that infected persons are capable of producing airborne virus does not necessarily indicate that virus can be transmitted in this way. Viral aerosols produced by infected persons are subject to dilution in room air, biological decay, and sedimentation. Nevertheless, assuming normal breathing by susceptible volunteers and an infectious dose of about 6 to 30 $TCID_{50}$, assay of air samples from rooms occupied by infected volunteers indicated that transmission would be accomplished in from 5 min to 24 hr. Furthermore, in view of the observed efficiency (11%) of the air-sampling equipment, larger than measured doses of virus were actually available for inhalation (14). In addition, the present data suggest that cough is a most important event in producing viral contamination of air.

The findings described above stimulated the performance of an experiment to test the assumption that airborne transmission is possible, and

preliminary results revealed that airborne transmission occurred from infected cases to susceptibles across a wire barrier.

Airborne and contact transmisison was simulated in volunteers by aerosol and nose drop inoculation, respectively. Studies with three different strains of coxsackievirus A type 21 indicated a similar HID_{50} of about 30 $TCID_{50}$ for this virus, when predominant deposition was in the lower respiratory tract (small-particle aerosol), and a lower value when nasal drops were used. Since the latter inoculation method provided deposition only in the nasopharynx, it is suggested that the nasal mucosa exhibited a greater susceptibility to infection with this virus than did the lower respiratory tract. Another picornavirus, rhinovirus NIH 1734, exhibited an even greater difference between the HID_{50} for nasal drop inoculation and for small-particle aerosol inoculation. Thus, the data suggest that, for both of these viruses, the nasal mucosa is the preferred site for infection. Although definitive comparisons are incomplete, present evidence suggests a disparity in infectivity in the opposite direction for adenovirus type 4. This virus exhibits a high degree of infectivity for the lower respiratory tract, but the nasopharynx appears to lack this degree of susceptibility.

The most common illness response to each virus that followed inoculation by nasal drops and small-particle aerosol is shown in Table 11. For comparative purposes, the most common naturally occurring illness response to each virus is also listed. As can be seen for coxsackievirus A type 21, regardless of method of inoculation as well as dose, febrile upper respiratory illness usually results in volunteers, whereas naturally occurring illness is reported to be usually afebrile (2, 18). This disparity may well be explained by the fact that fever in volunteers is usually so brief in duration that, without 24-hr observation, the majority of volunteers would have been designated afebrile. The predominant lower respiratory tract illness that was seen with one

TABLE 11. Characteristic natural and experimentally induced clinical responses to respiratory viruses

| Virus | Experimental inoculation | | Natural inoculation |
	Nasopharyngeal	Aerosol, 0.5 to 2.5 µ particles	
Coxsackievirus A type 21	Febrile URI	Febrile URI	Afebrile URI
Rhinovirus NIH 1734	Afebrile URI	Afebrile URI	Afebrile URI
Adenovirus type 4	Afebrile URI	Febrile URI or LRI, or both	Febrile URI or LRI, or both

Upper respiratory tract illness

inoculum administered by small-particle aerosol appears to have been relatively unique, and was due to properties of the virus in that inoculum that are not usually exhibited by strains of this virus.

For rhinovirus NIH 1734, afebrile upper respiratory tract illness occurs in volunteers regardless of inoculation method and is also the characteristic natural clinical response to this and other rhinoviruses (4, 10). Data thus far available indicate that naturally occurring adenovirus type 4 disease can regularly be reproduced in volunteers only by aerosol inoculation. Nasal inoculation, throat swabbing, and conjunctival inoculation have all failed to reproduce naturally occurring type 4 adenovirus disease (1).

It is therefore suggested that adenovirus type 4 disease is transmitted in natural circumstances primarily by the airborne route. The information available on coxsackievirus A type 21 and rhinovirus NIH 1734 indicates that either airborne or contact transmission would result in the upper respiratory tract illness characteristic of naturally occurring illness. However, the small-particle aerosol inoculation results suggest that airborne transmission would produce a more varied response and account for the lower respiratory tract illness which is sometimes associated with naturally occurring upper respiratory tract disease (4, 10, 18).

Thus, the data presented on production of airborne virus, environmental air contamination with virus, and the demonstration of airborne transmission summarized in the present report indicate that airborne transmission probably occurs naturally. Present information, however, does not indicate whether airborne transmission is the predominant mechanism of natural transmission. At the present time, it seems most reasonable to suggest that both contact and airborne transmission occur in natural circumstances, and that the predominant method of transmission varies with the virus and the opportunity presented in a particular situation. For those viruses and situations in which airborne transmission predominates, it may be possible to devise suitable methods of control of respiratory viral infection.

SUMMARY

Volunteers were inoculated with respiratory viruses by means of nasal instillations and inhalation of aerosols. The former method was used to simulate contact transmission, and the latter to simulate airborne transmission. The HID_{50} for coxsackievirus A type 21 was about 30

$TCID_{50}$ by aerosol and 6 $TCID_{50}$ by nose drops. Similar determinations for rhinovirus NIH 1734 revealed HID_{50} of 0.68 $TCID_{50}$ by aerosol and 0.032 $TCID_{50}$ by nasal drops. The clinical response was characteristically an upper respiratory tract illness for both viruses by both inoculation methods, although coxsackievirus A type 21 illness was usually febrile, and rhinovirus illness usually was not. Incomplete infectivity studies with adenovirus type 4 suggest a disparity in the opposite direction for this infection. Aerosol inoculation revealed an HID_{50} of about 1 $TCID_{50}$ and thus far is the only inoculation method which regularly reproduced naturally occurring ARD.

The suggestion that airborne transmission accounted for some naturally occurring acute respiratory disease was further evaluated by studying the production of airborne virus by coughs and sneezes and the contamination of room air with virus. Coughing and sneezing regularly produced quantities of virus sufficient to infect, whereas breathing did not. Room air samples revealed contamination probably sufficient to infect susceptibles. In addition, preliminary results of a transmission experiment with coxsackievirus A type 21 indicate that airborne transmission unquestionably occurred. It was concluded that both contact and airborne transmission of the respiratory viruses probably occur in natural circumstances, and that the predominant method of transmission may vary with the virus and with the particular environmental situation.

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Discussion

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The investigator of experimental infection has advantages—and, indeed, he *makes* these advantages—that the student of natural infection cannot have. He can operate with deliberation, he can pick his time and place, he can pick his subject, and his infecting agent (indeed, also its dose), he can control the environment and the route of inoculation, and he can observe the event we call infection both in prospect and in retrospect. Yet he lacks, and always will lack, the cardinal advantage possessed by the student of natural disease—for, unlike his colleague, he is not studying natural disease. This must never be forgotten. Dr. Couch and his associates are well aware of this point. Their interesting and provocative paper is a measured and careful analysis of basic investigations of an incredibly neglected aspect of medicine: the transmission of human respiratory infection.

There is much to discuss in this paper, as the authors themselves have discovered, and I should like to focus on the aspects of the study that seem to have broad implications for the pathogenesis and transmission of infection, rather than on the technical problems of air sampling and calculation of virus dose, although, to some extent, these matters are inseparable.

First, I would emphasize that the principal and best-documented part of this study concerns virus *input* and its effects rather than virus *output* and transmission. Commendably, the authors have recognized implicitly the inhomogeneity of "respiratory viruses" by their selection of three prototypes for study, and, further, have studied (with differing results) two strains of one prototype, coxsackievirus A21. This leads me to my first question. In Table 1, in which the data for determination of human infectious dose of coxsackievirus A21 are presented, we note that in contrast to the common phenomenon of infection without disease, we have disease without infection (after doses of 18 and 6 TCID$_{50}$)! This observation may be an important one and is not adequately explained by the notation that 15% of volunteers may have unexplained mild rhinitis, particularly since no control group is included in the experiment cited. Is this a nonspecific reaction, or is it rather a subtler measure of low-grade infection than the conventional indices of virus recovery and antibody response? A similar observation concerning experimental A21 injection has also been made by Buckland and

associates. If so, it is implied that the human and tissue culture infective doses may be close to unity. I do not wish to belabor an apparently trivial point, but we note that the virus used in this titration is the A21 strain, 49889, which is more virulent than the other A21 strain used, in its capacity to produce lower respiratory tract disease. Why this greater capacity to induce disease? Because of intrinsic difference in virulence of the virus in nature, because of the vagaries of virus selection during its isolation and passage, or because the human infectious dose has been miscalculated, with the result that a larger dose of this strain has actually been given? This type of problem will continue to beset us as we attempt to distinguish qualitative from quantitative factors in experimental infection. Incidentally, is the febrile course of experimental coxsackievirus A21 infection in contrast to the afebrile course observed in the field a reflection of selection of subjects with no antibody, or does it reflect differences in the conditions of infection?

But the striking fact that emerges from the studies of coxsackievirus A21 infection is the importance of the nature of the viral inoculum in determining the localization and severity of infection. The implication is clear that in the perhaps special case of the 49889 strain, lower respiratory tract disease requires initial implantation of virus at that site. It is also clear that one may have presumed implantation of virus in the lower respiratory tract without evidence of disease at that site, but with evidence of disease above. What a fine demonstration of the importance of host determination of clinical response in a precisely controlled situation. Restudy of such volunteers with an antigenically heterologous virus might confirm our strong suspicion that certain individuals are unduly subject to lower tract involvement in the course of upper respiratory tract infections. Now the other part of the question is: how does an aerosol of small particles produce upper respiratory tract disease? Probably not by extension from below as judged by the similar incubation periods of aerosol and nasal inoculation disease, but perhaps by the fraction of small particles that are retained in the upper tract. It must be kept in mind that there is a range of particle size with either method of inoculation.

The experiments with rhinovirus infection are also of interest and suggest again that a *sine qua*

mm for respiratory virus infection of the lowe· tract is initiation of infection by aerosol. The observation that a true common cold virus can indeed produce generalized respiratory disease is an important one. Incidentally, I disagree with the authors when they impute an all-or-none effect of pre-existing antibody in ameliorating disease. The number of cases is far too small (a chronic problem, incidentally, in volunteer studies) and the data suggest a graded response in terms of infection.

Turning to adenovirus infection, it is especially interesting that simulation of the natural disease is apparently dependent on infection by aerosol, despite the fact that its principal manifestations are in the nasopharynx. Influenced by our studies of influenza, Jerome Schulman and I have long contended that the site of predominant symptomatology is not necessarily an indication of the site of primary viral invasion. It is true, however, that natural infection with either influenza or adenoviruses may be associated with primary virus pneumonia, so the potential for that clinical manifestation is probably omnipresent.

In conclusion, we can note with great interest the brief mention by Dr. Couch and his associates of transmission of infection from man to man under conditions that exclude direct or indirect contact and that point to true airborne inoculation. The double wire screen used by Dr. Schulman with influenza in mice has now found application with man, coxsackievirus, and Quonset huts. We await further exploitation of this system by Dr. Couch and his colleagues with the imagination and thoroughness they have shown in the past.

The proper study of mankind may be the mouse. In an experimental mouse influenza virus model developed in our laboratory by Dr. Schulman, we have found that virtually all transmission of infection can be attributed to small-particle aerosols. This conclusion depends essentially on two types of observation: (i) that physical separation of contacts from transmitters exerted no effect on the transmission rate, and (ii) that increasing the rate of ventilation through the chamber where contact occurred decreased proportionately the rate of transmission. Both observations are inconsistent with transmission by larger droplets. Furthermore, in very recent experiments, we have recovered influenza virus from the air in proximity to transmitter mice in quantities that are virtually identical to the calculated output required to account for the observed transmission rate (a calculated 2.3 infectious doses per infector per 24 hr).

It is clear that experimental infection with a number of viruses may be initiated by any of several routes by either small or large particle inocula. It is now time to determine for each virus what, in fact, is its principal natural mechanism of transmission as it journeys from man to man. This can be done with the techniques now available, and, indeed, indirectly by such environmental controls as ultraviolet irradiation, employed 30 years ago by Wells.

Aerogenic Immunization of Man with Live Tularemia Vaccine

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INTRODUCTION

Vaccines are generally administered by the subcutaneous or intramuscular route. However, the immune response produced after parenteral administration is inadequate in many instances to ensure optimal host resistance. Many infectious diseases are acquired via the respiratory tree; possibly, the immunizing antigen would be more effective in inducing high-grade host defense if the route of administration were identical to the route of acquisition of the disease. Active immunization against airborne infection by inhalation of living, attenuated microorganisms has been proved with experimental animals and, in some instances, has become routine (1-4, 11-14, 16, 20, 22). The potential for immunization of man by aerogenic vaccination with single or combined live vaccines has been recognized in the Soviet Union (1-3) and in the United States (5, 8, 9). In the Soviet Union, vaccination of man with aerosols of dried, viable tularemia vaccine, singly or in combination with living vaccines of other microorganisms, has received considerable attention. Systemic reactions were reported by Alexsandrov et al. (2) in 2 of 138 volunteers inhaling an estimated 750,000 organisms contained in an aerosol of dried tularemia vaccine. Kerostovtsev, Onikiyenko, and Khokhlov (17) noted similar complaints in three of eight persons inhaling 7,500,000 cells of a comparable product. Immunity has been measured primarily by serological procedures and by reaction to skin test preparations, but has not been proved by increase in resistance of the vaccinee to challenge

with fully virulent organisms. Tigertt (23) has reviewed selected Soviet articles on viable tularemia vaccines, and Lebidinsky (18) has reviewed the published American literature on this subject.

In the United States, live tularemia vaccine prepared from *Francisella tularensis* strain LVS (live vaccine strain) (7) and administered percutaneously has been proved immunogenic and superior to killed vaccines in studies with volunteers by Saslaw et al. (21) and by McCrumb (19). Studies by the latter investigator revealed that, although immunized volunteers were protected against challenge by the respiratory route with 200 to 2,000 virulent organisms, resistance could be overcome in about 50% of men when the challenge dose was increased approximately 10-fold. (The genus *Francisella*, honoring the late Edward Francis of the U.S. Public Health Service and providing better taxonomy, will appear in the next edition of *Bergey's Manual*.)

In an effort to enhance the immunity provided by LVS, aerogenic vaccination was studied by Eigelsbach et al. (10, 11) and White et al. (24). It was demonstrated that this route of vaccination was not associated with untoward reactions, and only a mild, nongranulomatous response was observed in the respiratory bronchioles of monkeys that received aerosolized liquid vaccine. Animals so vaccinated evidenced excellent protection when challenged with virulent organisms.

In a recent unpublished study (H. T. Eigelsbach and J. J. Tulis) designed to determine the effect of aerosolized vaccine dose on reactivity and

TABLE 1. *Effect of dose and route of inoculation on the immunogenicity of live tularemia vaccine for the monkey*

Vaccination	Vaccine dose	No. of animals	Survival at 120 days after aerogenic challenge*
			%
Respiratory	10^7	16	94
Respiratory	10^5	15	60
Respiratory	10^3	17	47
Dermal	Acupuncture	16	81
Control	None	12	0

* With 10^4 cells of strain SCHU S4.

immunogenicity for monkey, groups of 15 to 17 *Macaca mulatta* inhaled 10^7, 10^5, or 10^3 cells of live tularemia vaccine strain LVS. Another group of 16 animals received LVS percutaneously by acupuncture; in this case, the actual number of cells introduced is unknown, because a substantial portion of the inoculum remains on the surface of the skin. Vaccination by either procedure proved innocuous, and resulted in comparable peak mean titers except in the aerosol group receiving the lowest dilution of organisms. Of the 17 animals that inhaled 10^3 organisms, 9 failed to develop agglutinins. The mean titer of the intradermal vaccinees rose earlier and faster than did titers of the aerogenic vaccinees. At 60 days after vaccination, these animals, as well as nonvaccinated controls, were challenged aerogenically with 10^4 cells of strain SCHU S4 (Table 1). All controls died within 30 days; 120 days after challenge, the per cent survival in the 10^7, 10^5, and 10^3 groups vaccinated aerogenically and in the group vaccinated dermally was 94, 60, 47, and 81, respectively. Because monkeys are less resistant to tularemia than is man, their benign response to aerosolized liquid LVS tularemia vaccine indicated that this vaccine might also be safe for man when administered aerogenically. Initial studies in volunteers indicated (9) that respiratory doses ranging from 200 to 30,000 organisms were innocuous and that approximately 1,500 inhaled cells were required to induce serological conversion consistently. These studies were expanded at the University of Maryland Research Ward at Jessup, Maryland House of Correction, and are the subject of the present report.

AEROGENIC VACCINATION OF VOLUNTEERS

Materials and Methods

F. *tularensis* LVS and highly virulent challenge strain SCHU S4 (6) were cultivated in a modified casein partial-hydrolysate liquid medium (R. C. Mills et al., Bacteriol. Proc., p. 37, 1949). Cultures, harvested after 16 hr of incubation with continuous shaking at 37 C, contained 35 × 10^9 to 40 × 10^9 viable organisms per milliliter. For aerogenic immunization with strain LVS or challenge with strain SCHU S4, aerosols were generated with a nebulizer that produced particles primarily in the range of 1 to 5 μ diameter. Methodology was comparable to that previously described by Griffith (15).

Prior to aerosolization of LVS for use as a vaccine in man, all available information pertaining to its safety was evaluated. Extensive experience gained in volunteers and laboratory workers at Fort Detrick by the acupuncture route (8) attested to the attenuation of this strain. Serious reactions, such as secondary pneumonitis or bubo formation, were not seen. Immunogenicity was evident from the excellent protection noted in vaccinated volunteers exposed to aerosol or intracutaneous challenge. The aforementioned thorough animal evaluations suggested that no untoward reactions were likely to occur in man after inhalation of LVS.

Clinical Reactions

Five groups totaling 253 volunteers free from tularemia agglutinins were exposed to aerosolized LVS. The dose ranged from 10^4 to 10^9 organisms. Reaction rates correlated directly with size of inoculum. After inhaling a dose of 10^4 LVS cells, about 30% of 42 volunteers had minor systemic complaints. The majority noted minimal upper respiratory symptoms, such as sore throat or slight cough. Practically all had pea-sized cervical nodes after exposure to aerogenic LVS. None had fever or roentgen evidence of pneumonic infiltration. The signs and symptoms were quite insignificant and would have been overlooked with casual examination or questioning.

A more severe reaction was associated with inhalation of a 10^9 inoculum. As a result of this massive dose, 90% of the volunteers were symptomatic with headache, coryza, chest pain, and malaise. Actually, they had mild typhoidal tularemia. In 80%, there were temperature elevations of >100 F which occurred on the average at 3 days and lasted an average of 2.5 days. Of 42 men receiving this huge dose, 3 were treated with streptomycin, and several others were put to bed for periods of 2 to 3 days. Chest X rays revealed transient infiltrations in a few of the vaccinees. In general, the reaction just described can be likened to a "flu-like" syndrome. This condition did not incapacitate the majority of volunteers; they were able to con-

tinue their prison routine. Similar, but milder reactions, were seen in 79%, of volunteers inhaling 10⁴ cells of LVS

Serological Response

Results of serological studies have shown the correlation between size of inhaled dose (antigenic mass) and acquisition of serum agglutinins. Volunteers receiving the largest number of organisms developed demonstra le serum antibodies in a surprisingly short time. By the second week postvaccination, 92%, of the volunteers had agglutinins, and at 3 weeks a 97%, incidence was recorded. This rapid acquisition of serum antibodies following the large inhaled antigenic mass was more rapid than the rate following LVS administered by acupuncture (65%, at 2 and 82%, at 3 weeks). The preliminary studies with smaller aerogenic inocula revealed a delayed response when compared with the intracutaneous route of vaccination. The results of these series of investigations suggested that large groups of nonimmune people can be immunized more rapidly by the respiratory than the intracutaneous route; however, a high incidence of systemic reactions would result from exposure to large-dose vaccine aerosols. Although there is more rapid seroconversion noted with the latter method (large-dose aerosol), the geometric mean titers were no different after 8 weeks whether vaccination was accomplished by acupuncture or with smaller-dose aerosols.

Conversion rates were reduced and geometric mean agglutinin titers were delayed as the inhaled dose was lowered. After the 10³ log dose of LVS, geometric mean titers did not begin to rise significantly until the 3rd week postvaccination, and antibody levels comparable to those associated with acupuncture vaccination occurred between the 4th and 5th weeks (Fig. 1). Both geometric mean titers lagged behind those of the two largest aerogenic vaccine doses. Similarly,
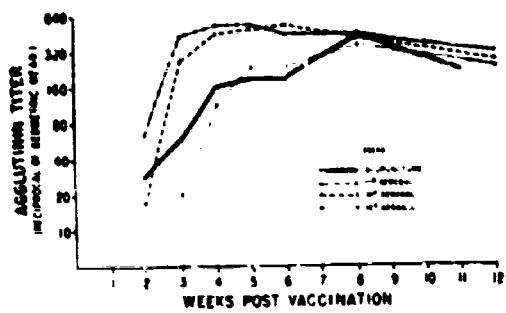
Fig. 1. Agglutinin response to LVS vaccine administered in varying doses by the aerogenic route compared with response after intradermal inoculation

Table 2. Response of volunteers to large dose respiratory challenge after aerogenic vaccination

Interval between vaccination and challenge	No. total challenge	No. with fever over 102 F	No. requiring therapy	Per cent protected (men)
(months)				
2	22	15	6	73
4	30	18	0	100
6	16	10	0	100
14	32	26	16	50
18	2	2	1	50
Summation, 2-18	102	71 (70%)	23 (23%)	77
Controls	47	44 (94%)	42 (89%)	

ᵃ With 2.5 × 10⁴ organisms, strain SCHU S-4.
ᵇ Criterion for treatment was 103 F or greater for over 24 hr.
ᶜ Uncorrected with respect to control data.

seroconversion rates peaked at the 90%, level 5 weeks postvaccination, compared with 3 weeks. Nevertheless, geometric mean titers eventually reached antibody levels achieved with larger aerosol doses.

CHALLENGE OF AEROGENIC VACCINEES WITH VIRULENT F. TULARENSIS

Aerogenic Challenge

The presence of circulating tularemia agglutinins is not tantamount to resistance to the disease. It remained, therefore, to evaluate the degree of protection of the volunteers to challenge with virulent F. tularensis. Table 2 outlines the results of aerogenic challenge with 2.5 × 10⁴ organisms. This challenge represents approximately 2,500 times the minimum infective dose for man, which has been estimated to be 10 to 50 organisms (21). This was a severe challenge and probably far exceeds the number encountered during natural exposures. In this experiment, the interval between vaccination and time of challenge did not appear to be a determining factor in the extent of protection. At 2 months 73%, of 22 and at 14 months 50%, of 32 volunteers exposed developed disease and were treated with antibiotics. (The difference was not significant by the chi square test.) These two groups received the same dose of aerosolized vaccine. Those men challenged at 4 and 6 months received the two highest doses of LVS (10⁴ and 10⁵), and the subsequent mild vaccine infection may have contributed to the excellent overall resistance of the groups.

Table 3 illustrates the significance of the method of vaccination of these volunteers in relation to

TABLE 3 Relationship of route and dose of LVS vaccine to resistance to tularemia aerosol challenge

Dose	Route	No. chal- lenged	No. % with fever -loss F[b]	Per cent respon- ing treat- ment[c]	Per cent pro- tec- tion[b]
10[9]	Respiratory	30	18 (60)	0	100
10[6]	Respiratory	16	10 (62)	0	100
10[5]	Respiratory	56	43 (77)	41	50
	Dermal	46	29 (63)	46	54
	None	47	44 (94)	89	

[a] Mean incubation period volunteers vaccinated aerogenically or by acupuncture, approximately 4 days, controls, approximately 3 days

[b] Uncorrected with respect to control data

[c] Acupuncture technique.

TABLE 4 Response of volunteers to large dose respiratory challenge after acupuncture vaccination[a]

Interval between vaccination and challenge	No. chal- lenged	No. with fever -loss F	No. requiring therapy[b]	Per cent pro- tec- tion[c]
months				
2	5	1	0	100
4	13	10	4	69
11	8	6	6	25
14	19	11	10	47
36	1	1		1
Summation				
2-36	46	29 (63),	21	54
Controls	47	44 (94),	42 (89%)	

[a] With 2.5 × 10^{5} organisms, strain SCHU S4.

[b] Criterion for treatment. 103 F per os or greater for over 24 hr.

[c] Uncorrected with respect to control data.

resistance to respiratory challenge. High-grade protection was acquired by the men inhaling 10[9] or 10[6] doses of LVS. A somewhat lower grade protection was observed in man immunized aerogenically with 10[5] LVS or by acupuncture; similar protection resulted in both groups. The incidence of infection in all four groups of vaccinees was equivalent (60 to 77), had fever of 100 F or greater), but the incidence of the disease was quite different. The 28 infected men in the two groups who had received large doses of LVS by the aerogenic route reacted to the initial infectious process developing from the severe respiratory challenge, but the acquired resistance prevented progression to overt disease requiring specific treatment.

The average incubation period for the control subjects was less by 1 day than that of the vaccinees. The shorter incubation period in the controls plus equal incubation time for all vaccinees, acupuncture as well as aerogenic, suggests that respiratory exposure to LVS did not sensitize the lung parenchyma. If a hypersensitivity reaction had occurred in men vaccinated aerogenically, immediate febrile or systemic reactions might have been expected. No evidence of such reaction was observed.

Table 4 presents data accumulated from additional experiments wherein volunteers, immunized by the acupuncture technique, were challenged aerogenically at varying intervals postvaccination. Although the numbers of subjects were small, results were similar to those observed after small-dose aerosol LVS. Immunity waned at about 1 year to the same extent.

Unvaccinated volunteers without demonstrable tularemia agglutinins served as controls in these aerosol challenges. Five of 47 men failed to

develop disease. Actually, four men represent these five failures; two were rechallenged and developed pneumonic tularemia, a third was re-exposed on two additional occasions before disease was induced, and the fourth has not been rechallenged. Each appeared to be a complete "miss" at time of exposure without subsequent subclinical infection, because antibodies were not demonstrable. Mechanical difficulties, i.e., loose-fitting masks, were implicated as the significant reasons for failure to produce disease and not natural host resistance, because of susceptibility to rechallenge. Similar incidents may also have occurred in the exposed vaccinees. The low frequency of "misses" and presumed equal distribution would not invalidate the per cent protection observed in the challenged vaccinees.

Intradermal Challenge

Small numbers of volunteers receiving vaccine by the respiratory route have been challenged by the intradermal inoculation of 1,000 to 10,000 infectious doses per man of SCHU S4 strain (Table 5). Protection was excellent. Not only was there no evidence of lessened immunity after 6 months, but also resistance to massive challenges was uniform. The disease rates were comparable to those following challenge of volunteers vaccinated by acupuncture. The clinical appearance of inoculation sites was strikingly different from the lesions in controls. The skin lesion resembled a delayed hypersensitivity reaction in the immune individual; control

TABLE 5. *Response of vaccinated volunteers to intradermal challenge*

Type of vacci-nation	Challenge dose (organisms)	Interval be-tween vacci-nation and challenge	No. chal-lenged	No. re-quiring therapy[a]	Number protected/total	Controls requir-ing therapy total
		months				
Acupuncture	1 × 10³	6	10	1	9-10	10-10
	8 × 10³	6	8	1	7-8	1-1
	1 × 10⁴	2	3	1	2-3	2-2
	1 × 10⁶	2	3	1	2-3	
Total	3 × 10³-1 × 10⁶	2-6	24	4	20/24 (83%)	
Aerogenic	1 × 10³	2	7	0	7/7	
	1 × 10³	6	4	0	4-4	4-4
	1 × 10⁶	2	3	2	1-3	
Total	1 × 10³-1 × 10⁶	2-6	14	2	12/14 (86%)	

[a] Criterion for treatment, development of a typical ulceroglandular infection similar to that in controls.

subjects showed progressively developing ulcers. Based on this small experience, it appears that aerosolized LVS produces effective immunity to ulceroglandular tularemia.

Relationship of Antibody Titers to Immunity

Analysis of agglutinating antibody titers in the vaccinees suggests that higher levels were associated with less severe illness, and groups of inmates requiring treatment had baseline geometric mean titers one-half the value of those groups not treated. On the other hand, absence of disease in the respiratory challenge group which received the massive dose of aerogenic LVS cannot be explained solely on the basis of elevated titers. Geometric mean titers in this group were equivalent to those of the other aerogenically vaccinated groups. Challenge results were quite different; 6 of 22 men had disease when exposed 2 months after small-dose aerosol vaccination, but none of 30 men had disease following challenge at 4 months after large-dose vaccine aerosol. Thus, although absolute level of agglutinins cannot be correlated with immunity, presence of these antibodies in the sera of volunteers exposed to virulent challenge suggests that members of the group will resist infection to a greater degree than unvaccinated controls.

DISCUSSION

Immunization of man against tularemia can be accomplished safely by employing aerosolized living attenuated vaccine. The dose necessary to ensure development of serum antibodies in at least 90%, of volunteers is 10⁴ organisms. Systemic subjective reactions at this dose were not significant, and close clinical observation was necessary to reveal subtle objective findings, i.e., appearance of pea-sized cervical lymph nodes.

The inhaled dose can be increased without undue risk if more rapid induction of antibodies is desired. As many as 10⁹ organisms have been delivered to volunteers as an immunizing dose. Low-grade febrile disease occurred in more than 90%, of the volunteers with this dose. However, the reaction was mild and self-limiting, and did not interfere with the daily routine of most inmates. After this vaccination, a high-grade immunity was observed against a severe aerogenic challenge conducted 4 months after vaccination. Assurance is provided, thereby, that, even if unlikely dilution errors would create such concentrated aerosols, exposed healthy young adults would experience only mild discomfort. Acceptability of this aerosolized antigen is questionable in people with chronic lung disease, congestive failure, or other diseases affecting the integrity of respiratory defense mechanisms. Perhaps small doses of aerosolized LVS could be tolerated in such patients. Sufficient evidence bearing on this point is unavailable.

These studies validate the respiratory route as a means of introducing an attenuated bacterium into the human host. It remains to be determined whether this route is more advantageous than the conventional dermal site. Aerosolized vaccine does lead to an immune state. The incidence of disease after challenge of volunteers vaccinated by this method was less than that recorded in the men challenged after immunization by acupuncture. This difference occurred primarily in groups that received the larger doses of aerosolized LVS. These men had mild tularemia after vaccination, and the virulent challenge can

almost be considered a rechallenge. This type of immunization provoked more resistance to infection. Circulating antibodies alone are not sufficient to explain differences in protection; the geometric mean titers were identical for the aerogenic vaccine groups challenged at 2 and 4 months, respectively; yet, disease rate was greater in the former. Perhaps the lower disease rate results from the ability of lung tissue previously exposed to LVS to confine better the inhaled pathogens through better phagocytosis, tissue antibody effect, or other local defense mechanisms. Thus, it seems reasonable to expect that in respiratory-acquired infectious diseases prior vaccination with sufficient antigen given by the aerogenic route will produce increased host protection. Present evidence is insufficient to allow conclusions regarding the protection afforded aerogenically vaccinated individuals against the ulceroglandular form of tularemia. Following the reasoning above, the acupuncture method should be the best way to prevent this disease. The differences in distribution of vaccine by the two routes into the two organs initiates dissimilar reactions for developing local tissue defense. Therefore, analogous reasoning cannot be applied to the skin.

One disadvantage of the aerogenic vaccination technique is the lack of a "marker" indicating vaccine reaction. The scar from the acupuncture route is obvious for weeks. Nevertheless, the need for visible evidence of reaction to vaccine is lessened when over 90% of an exposed population are immunized by simple inhalation of LVS. In addition, serological proof of vaccination is easily obtained.

The elaborate exposure equipment used in these studies allowed for precision in uniformity of particle size and quantitation of the inhaled dose. The application of aerosolized vaccines on a mass basis will require simple, less complicated apparatus. Efforts to create such instruments should be encouraged. Soviet literature contains reference to mass aerogenic vaccination of troops exposed in tents (6). Vaccination by the respiratory route for tularemia is effective, and this fact should serve as an impetus for future experimental studies with viral and bacterial vaccines.

SUMMARY

Live, attenuated LVS tularemia vaccine has been administered via the respiratory route in doses ranging from 10^4 to 10^9 organisms. Mild self-limiting typhoidal tularemia was induced by doses of 10^4 to 10^9 vaccine organisms. Rapidity of induction of agglutinin titers in the human host varies directly with size of inhaled inoculum. Immunity to aerogenic virulent *F. tularensis* challenge appeared to be greater than that produced by the conventional acupuncture method of vaccine administration. Protection against ulceroglandular tularemia was also demonstrated. The pulmonary tree in man can be safely and successfully utilized for application of *F. tularensis* strain LVS and possibly for other microorganisms.

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Respiratory Antibody to *Francisella tularensis* in Man

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For several years my colleagues, J. Bellanti and M. Artenstein, and I have studied the occurrence of specific antibodies in secretions of the respiratory tract, and have attempted to evaluate their biological significance (2, 3, 3a). This interest stemmed from the need for a simple biological marker to identify persons most likely to resist overt respiratory infection with any of several viruses. As with respiratory tularemia, the presence or titer of *humoral* antibodies to respiratory viruses is *not* synonymous with resistance to clinical disease upon infection. When these investigations were begun, it was our purpose to identify such markers. We chose to reinvestigate the occurrence of local antibody in respiratory secretions by use of more modern virological and immunological methods. Such antibodies were indeed found in nasal secretions of normal individuals. Not every individual possesses antibody to each respiratory virus; rather, detectable antibodies occur in patterns which varied from person to person (2, 3). Although there is no doubt that local antibody exists in the respiratory tract, little is known of its influence upon the pathogenesis of respiratory infections. Recently, we studied respiratory antibody to *Francisella tularensis* in man; these observations are pertinent to the questions raised by Drs. Hornick and Eigelsbach concerning effectiveness of aerosol immunization against respiratory tularemia.

Last year, with H. Dangerfield and D. Crozier of the Medical Unit, Fort Detrick, Frederick, Md., we studied respiratory antibody in 14 volunteers before and after aerosol infection with virulent *F. tularensis* (SCHU-S4 strain); this investigation will be reported in detail elsewhere. Eight volunteers were immunized percutaneously 3 months previously with LVS (tularemia vaccine, live attenuated) vaccine containing approximately 10⁸ viable LVS cells per 0.1 ml. Six served as susceptible controls. One-half of each group was challenged by aerosol containing approximately 2,500 organisms; the other, with 25,000 cells. Nasal secretions were collected from these individuals by previously described methods (2) twice daily for 3 days before and for 5 days after challenge, and at weekly intervals thereafter for 6 weeks. Daily collections of nasal washings from each volunteer were pooled, concentrated approximately 10-fold by lyophiliza-

tion after dialysis against distilled water, and studied for hemagglutinating antibody to polysaccharide prepared from the SCHU-S4 strain. Antibody determinations were made by the method of Alexander (1) modified for microtiter technique. Hemagglutinating antibody was measured because it is more readily detected in higher titers than are cell agglutinins (4).

Nasal antibody was indeed detected 3 months after percutaneous immunization, prior to challenge by aerosol infection (Table 1). Titers of nasal antibody ranged from 1:2 to 1:32 per 0.05 ml of concentrated nasal washing, and, for the most part, were significantly lower than those observed simultaneously in serum. There was no clear correlation between titers of antibody in serum with those found in secretions (Table 2), although too few individuals were studied to make absolute comparisons. However meager, the

TABLE 1. *Occurrence of serum and nasal antibody in eight persons to Francisella tularensis 3 months after percutaneous immunization*

Determination	Antibody[a] titer								
	2	4	8	16	32	64	128	256	512 or >
Nasal washings	3[b]	1	2	1	1				
Serum					1			2	5

[a] Reciprocal per 0.05 ml.
[b] Results expressed as number of persons with indicated titer.

TABLE 2. *Correlation between serum and nasal antibody titers 3 months after percutaneous immunization*

Nasal antibody[a]	Serum antibody[a]				
	8	256	512	1,024	2,048
2	•	•	•		
4				•	
8			•		•
16				•	
32		•			

• Hemagglutinin per 0.05 ml. At serum antibody dilution of 1:16 through 1:128, no hemagglutination occurred.

TABLE 3. *Development of nasal and serum antibody after aerosol infection with Franciscella tularensis*

Challenge dose	Patient no.	Determination	Titer[a] pre-exposure	Titer[a] at indicated day postexposure					
				1	3	7	14	21	42
±2,500 cells	1	Serum	<2	—	—	<2	256	1,024	—
		NW[b]	<2	<2	<?	2	16	32	32
	2	Serum	<2	—	—	2	32	4,096	16,384
		NW	<2	<2	<2	<2	2	128	—
±25,000 cells	3	Serum	<2	—	—	<2	64	128	256
		NW	<2	<2	<2	<2	8	8	16
	4	Serum	<2	—	—	<2	64	512	512
		NW	<2	<2	<2	<2	4	16	8

[a] Per 0.05 ml of serum or nasal washing.
[b] NW = nasal washing.

data suggest that detectable nasal antibody occurred in persons with serum antibody titers of 1:256 or greater. The quantitative relationships between titers of nasal and serum antibodies remain to be determined.

Susceptible volunteers, when exposed to aerosols containing either 2,500 or 25,000 living cells, similarly developed nasal antibody (Table 3). Antibody was detected as early as 7 days after exposure (patient no. 1), was regularly present at 14 days, and increased in titer to levels essentially similar to those observed in percutaneously immunized personnel 3 months after vaccination (1:8 to 1:32), except for patient no. 2, whose nasal antibody titered 1:128 on the 21st day after infection. Again, titers were significantly lower than those observed simultaneously in serum. Each of the four individuals experienced respiratory tularemia, and was treated with antibiotics in the conventional fashion (6). Thus, it is clear that, irrespective of the method for infection, human beings develop nasal antibody to *F. tularensis.*

This hemagglutinating antibody of nasal secretions was found to be associated primarily with γA immunoglobulin components. Antibody-bearing secretions from each of two individuals, either the result of immunization or infection, were absorbed with goat antisera against human γA and γM immunoglobulins (Table 4). Absorption with antihuman γA immunoglobulin removed all hemagglutinin from each secretion; in contrast, absorption with antihuman γM immunoglobulin failed to remove significant amounts of antibody. Further, nasal antibody appeared to be significantly different from that of serum in the same individuals (Table 5). When high titered postimmunization or postinfection sera and nasal washings were subjected to gel filtration (Sephadex G-200), patterns of eluted

TABLE 4. *Removal of hemagglutinin from nasal secretions by specific absorption*

Antibody induced by	Subject	Antibody titers after absorption with		
		Nothing	γM	γA
Immunization	1	8	4	<2
	2	8	8	<2
Infection	1	8	4	<2
	2	8	2	<2

hemagglutinin differed between serum and secretions. The majority of antibody activity in serum was associated primarily with the γM immunoglobulins, whereas nasal antibody was found primarily in eluates containing γA immunoglobulins, and this pattern was the same after either percutaneous or aerosol infection.

These observations show that there is no significant difference in the nature of local or humoral distribution of hemagglutinin to *F. tularensis* between persons infected percutaneously or by the respiratory route. If this antibody in any way reflects resistance to overt infection (and there certainly are reasons to question this assumption), it may be properly concluded that such differences as might be effected by varying the route of vaccine administration would be only chronological. Hornick and Eigelsbach showed that the humoral antibody response following aerosol immunization is more rapid than the response to percutaneous vaccination (5). Whether local antibody appears in the respiratory tract less rapidly after percutaneous immunization is, of course, unknown, but is readily subjected to test in percutaneously immunized volunteers.

Even if respiratory antibody appears more

TABLE 5. *Partition of nasal and serum antibodies to Francisella tularensis by Sephadex gel filtration*

Sample,* mode infection (native titer)		γM → γA → γG										
Serum pv (256)	0	2	16	8	8	2	1	0	0	0	0	
NW pv (32)	J	0	1	1	2	0	0	0	0	0	0	
Serum pi (4096)	0	4	64	64	32	16	8	4	2	0	0	
NW pi (128)	0	0	0	0	4	2	0	0	0	0	0	
Cumulative eluate vol (ml)	35	40	45	50	55	60	65	70	75	80	85	90

*NW = nasal washing. The serum samples both contained immunoglobulin components γM, γA, and γG; the NW samples contained γA and γG.

promptly after aerosol immunization, there are few circumstances which demand this extraordinarily prompt immune response. Further, it is clear that, despite the presence of respiratory antibody, the immunity induced by any method of immunization can be overwhelmed by challenge with more than 10,000 virulent cells. Finally, Hornick's experience shows that administration of LVS vaccine by aerosol is not without risk of reaction (5). Indeed, to obtain optimal protection for up to 6 months, it appears necessary to administer over 10^4 to 10^8 viable vaccine cells. Approximately 80% of those receiving these doses of vaccine had, as a reaction, overt but mild respiratory tularemia. This appears to be a greater price for an additional short interval of immunity than we would be willing to pay.

Finally, it is clear that this experimentation is seriously limited by the lack of a good reproducible marker for immunity (resistance to overt infection). It is not now possible to evaluate local antibody as a marker for immunity to *F. tularensis*, although in one other respiratory infection there appears to be a good correlation between presence of respiratory antibody and resistance to infection. Experiments with parainfluenza virus type 1 in man show that persons with nasal neutralizing antibody are more resistant to challenge infection than are those without, irrespective of their humoral antibody status (Smith et al., New Engl. J. Med. *in press*). Thus, recent experimental evidence strongly suggests that detailed analysis of respiratory secretions may well provide better markers for immunity to respiratory infections. This experimental approach is not technically difficult today, and should be extended further into the problem at hand.

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Antibiotic Prophylaxis and Therapy of Airborne Tularemia

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INTRODUCTION

Streptomycin was the first effective antibiotic for the therapy of tularemia, and remains the drug of choice (1, 14, 21). Alternatives are needed, however, because of (i) the possibility of infection by streptomycin-resistant *Francisella tularensis* (11), (ii) the need for injection of streptomycin with the attendant inconvenience and discomfort, and (iii) the toxicity of streptomycin. Of the many other antibiotics active against *F. tularensis*, the best evaluated and most frequently used are the tetracyclines and chloramphenicol (2, 9, 11, 12, 20). (Because chloramphenicol offers no advantages over the tetracyclines in the treatment of tularemia and has significant toxicity, only the tetracyclines will be considered hereafter except in reviewing earlier work.) Patients with acute tularemia respond well to therapy with either streptomycin or tetracycline; symptoms rapidly remit, and defervescence is prompt (Fig. 1). The late consequences of treatment with the two antibiotics differ, however. Relapses rarely follow exhibition of reasonable doses of streptomycin but occur frequently after therapy with conventional regimens of tetracycline (2, 11). Such relapses result from the persistence of bacteria in the tissues, not the emergence of tetracycline-resistant organisms; retreatment with tetracycline is effective (Fig. 1).

In addition to their use in the management of tularemia, antibiotics may be employed for prophylaxis, used here to mean treatment instituted during the incubation period to prevent illness. Results have been similar to those

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achieved in the therapy of acute disease: streptomycin prevents illness, but broad-spectrum drugs merely delay disease. McCrumb et al. (9), for example, consistently protected volunteers by administration of streptomycin for 5 days after intradermal inoculation with *F. tularensis*, whereas only two of five volunteers were protected from tularemia by 5 days of prophylactic treatment with chloramphenicol.

Comparison of their actions against *F. tularensis* in vitro may help to explain the difference in effectiveness of streptomycin and tetracycline in both the prophylaxis and therapy of tularemia. Streptomycin is bactericidal in vitro, and may eradicate the organisms without the intervention of host mechanisms. Tetracycline, even in high concentration, merely suppresses multiplication; organisms persist in the tissues until destroyed by host defenses. *F. tularensis*, like other intracellular pathogens (5,17), is cleared from the cells slowly even when multiplication is prevented, e.g., by a bacteriostatic antibiotic. The relative inefficiency of host defense against *F. tularensis* is a crucial factor in determining the effectiveness of prophylaxis and therapy of tularemia with bacteriostatic agents. In the Conference on Airborne Infection held in 1960, McCrumb cited the imperfect results achieved with bacteriostatic drugs and suggested that either prolonged or intermittent treatment might be required if they were to be completely effective (10). The success of such regimens in other intracellular infections, e.g., scrub typhus (8, 16) and Q fever (18, 19), prompted the present studies of tetracycline prophylaxis and therapy of experimental airborne tularemia in *Macaca mulatta* and man.
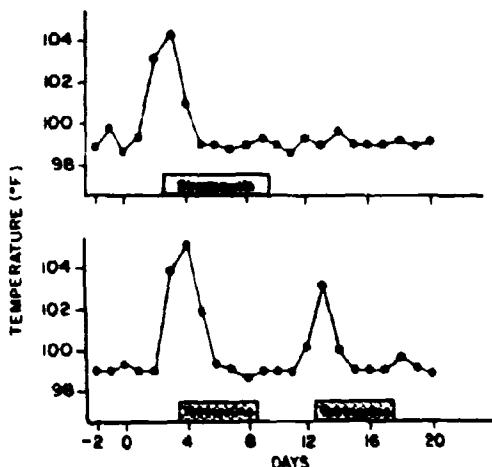
FIG. 1. *Therapy of experimental human airborne tularemia with streptomycin and with tetracycline. Doses were: streptomycin, 1 g twice daily; and tetracycline, 0.5 g four times daily.*

TETRACYCLINE PROPHYLAXIS

Simian Tularemia

Monkeys were exposed to aerosols of *F. tularensis* SCHU-S4 generated in a modified Henderson apparatus (3, 4, 7). [Healthy young adult *M. mulatta*, weighing 3 to 6 kg, were obtained from the Animal Farm, Fort Detrick, Md. Pre-exposure sera did not contain *F. tularensis* agglutinins. Cultures of *F. tularensis* were kindly supplied by H. T. Eigelsbach. They were grown in modified casein hydrolysate medium (Mills et al., Bacteriol. Proc., p. 37, 1949) for 16 hr with continuous shaking at 37 C, and were stored at 4 C until used. The SCHU-S4 strain is sensitive to streptomycin.] The average inhaled dose was 10,000 organisms, a quantity regularly resulting in an acute fatal illness after a short incubation period (Fig. 2). [In addition to twice daily examination and thermometry, serum C-reactive protein was determined and a chest X ray was obtained at weekly intervals (or more frequently upon request of the attending veterinarian). Fever (rectal temperature > 40 C) was the principal criterion of illness.] The results of five schedules of tetracycline prophylaxis are shown in Table 1. In all schedules, the initial dose of drug was given 24 hr after exposure, and prophylactic treatment lasted for 13 days. Illness was suppressed in 10 of the 11 animals receiving the antibiotic at 24- or 36-hr intervals; an unrelated, intercurrent illness cannot be excluded in the one exception. When the interval between doses was increased beyond 36 hr, however, the

animals experienced one or more febrile episodes during the treatment period. Because tetracycline administered at 48-hr intervals failed to suppress disease, a different sort of interrupted schedule was tried, i.e., 3-day treatment periods alternating with 2 day periods without drug. Four of the six monkeys were ill during the prophylactic period. Frequent administration of tetracycline, therefore, appeared necessary to limit multiplication of *F. tularensis* so that the infection remained subclinical during the treatment period.

After completion of all of the prophylactic regimens, most of the monkeys became ill (Table 1). Clearly, *F. tularensis* had remained viable in the host tissues throughout the period (13 days) of antibiotic administration. That the duration of persistence could be quite prolonged was demonstrated in another group of monkeys which received tetracycline daily for 6 weeks. Monkeys tolerated prolonged tetracycline treatment well, i.e., weight was maintained and no illnesses
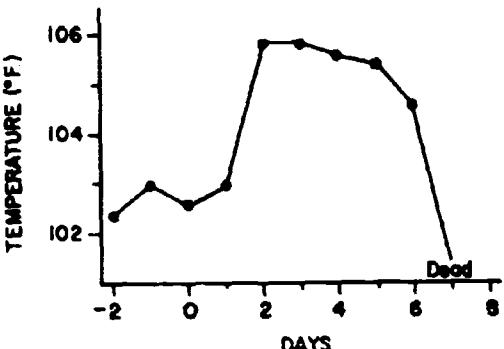
FIG. 2. *Course of fever in experimental simian airborne tularemia.*

TABLE 1. *Tetracycline prophylaxis of airborne tularemia in Macaca mulatta*[a]

Dosage interval	No. of doses	No. of monkeys	No. ill during treatment	No. ill after treatment	No. of deaths
hr					
24	13	5	1	5	0
36	9	6	0	4	1
48	7	6	6	6	1
72	5		6	6	0
Intermittent[b]	9	6	4	2	0

[a] Each animal received 200 mg of tetracycline intragastrically beginning on day 1 and continued over a period of 13 days. Six of six untreated animals developed fatal tularemia.

[b] Days 1 to 3, 6 to 8, 11 to 13.

attributable to the drug or to "superinfection" were detected. All remained well throughout the treatment period, but two of the six animals developed acute tularemia within 6 days of the last dose of drug. Because rigid precautions were taken to prevent re-exposure to *F. tularensis*, e.g., cross-infection, accidental laboratory aerosol, etc. (6, 7), these illnesses are believed to have resulted from organisms which were not eliminated during the 42 days of tetracycline treatment.

Even with treatment once a day, tissue levels of tetracycline undoubtedly fluctuated considerably, and, when levels were lowest, the organisms might have undergone several cycles of multiplication without yielding a bacterial mass sufficient to produce illness. This seemed unlikely, because agglutinins did not develop in monkeys who remained well during the course of daily prophylaxis (Fig. 3). When these animals became ill after cessation of treatment (see above), agglutinins promptly appeared. Agglutinin titers increased early in monkeys receiving prophylaxis which failed to suppress illness.

Although prophylactic treatment of tularemia with tetracycline failed to prevent illness, it reduced the severity of the disease. Whereas all of the six untreated monkeys died of tularemia, only two of those in the several prophylaxis groups expired within 70 days of exposure, the duration of observation (Table 1).

Because the timing of the institution of treatment may have important bearing on the effectiveness of prophylaxis of intracellular infection [e.g., tetracycline prophylaxis of Q fever merely
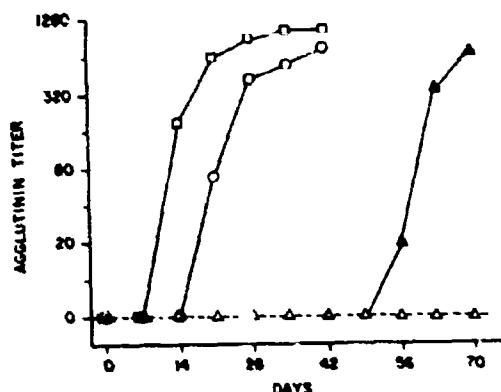
FIG. 3. Mean *Francisella tularensis* agglutinin titers of *Macaca mulatta* receiving tetracycline prophylaxis for airborne tularemia. Symbols: □ drug every 48 hr, 6 doses; ○ drug daily, 13 doses; △, drug daily, 42 doses, animals remaining well; ▲, drug daily, 42 doses, animals becoming ill after treatment.

TABLE 2. *Tetracycline prophylaxis of airborne tularemia in Macaca mulatta - Delayed institution of treatment*[a]

Treatment instituted (hr postexposure)	No. of monkeys	No. ill during treatment	No. ill after treatment	No. of deaths
24	5	1	5	0
60	6	1	6	2

[a] Each animal received 300 mg intragastrically once daily for 13 doses.

delays illness if instituted early but is preventive when begun during the last half of the incubation period (18, 19)], initiation of prophylactic treatment was delayed until 60 hr after exposure to *F. tularensis* in one group of monkeys. The results were no better than those obtained with earlier treatment (Table 2). Further delay in initiating prophylaxis was not feasible, because most monkeys became ill between 60 and 72 hr after exposure.

Human Tularemia

The failure of prolonged tetracycline prophylaxis to prevent simian airborne tularemia results from the limited defenses of *M. mulatta* against *F. tularensis*. Although man is quite susceptible to infection with airborne *F. tularensis*, he has better defense mechanisms than *M. mulatta*; e.g., the human respiratory infectious dose is about three times that of monkeys (15), and untreated airborne tularemia has a mortality of less than 50% in man (13) but is usually fatal in monkeys. It seemed likely, therefore, that prophylactic regimens of tetracycline which were only partially successful in monkeys might succeed in man.

The results of trials in volunteers who inhaled 25,000 *F. tularensis* SCHU-S4 confirmed the prediction (Table 3). [Healthy young Seventh Day Adventist soldiers participated on a voluntary basis; they were informed of the nature of the studies prior to volunteering (Army Regulation 70-25, Use of volunteers as subjects of research). The men were observed closely in the hospital before and after exposure. Sera obtained prior to participation did not contain *F. tularensis* agglutinins. The volunteers were examined at least twice daily, and their rectal temperature was recorded every 6 hr. Blood count, erythrocyte sedimentation rate, and serum C-reactive protein were determined and a chest X ray was obtained weekly (more often during periods of illness). Fever (rectal temperature >37.8 C), unassociated with signs of a disease other than tularemia, was the principal criterion of illness.

TABLE 3. *Tetracycline prophylaxis of human airborne tularemia (treatment instituted 24 hr after exposure)*

Daily dose	Frequency	Duration	No of subjects	No ill during treatment	No ill after treatment
		days			
1	Daily	15	10	0	2
1	Daily	7*	8	0	0
2	Daily	14	8	0	0
1	Every 2nd day	19	8	2	8

* Divided into morning and evening doses.

Volunteers who developed disease after completion of an experimental schedule of tetracycline were promptly treated with streptomycin, 1 g each 12 hr for 14 doses. All recovered quickly without complications or sequelae. Aerosols of *F. tularensis* were created in a modified Henderson apparatus (3, 4). The men inhaled through the nose and exhaled through the mouth.] All control subjects developed acute tularemia between 2 and 7 days after exposure. (These men participated in studies of therapy; see below). Administration of 1 g of tetracycline each day, beginning 24 hr after exposure, completely suppressed illness during the treatment period, but when treatment was stopped after 15 days, 2 of 10 volunteers developed acute tularemia. Extension of treatment to 28 days prevented illness. Complete protection was also achieved by administration of 2 g of tetracycline daily, even though treatment was terminated after 14 days. Intermittent drug administration, i.e., every other day, failed to protect the volunteers. The pattern of agglutinin response was consistent with the clinical effectiveness of prophylactic therapy, i.e., titers were high (1:1,280) in subjects after overt illness but negative or low (1:80 or less) in the men who remained free from disease.

In contrast to the results in *M. mulatta*, the human studies showed that satisfactory prophylaxis of airborne tularemia could be achieved with tetracycline, the simplest and shortest regimen being 2 g of drug daily for 14 days. With this schedule, disease was completely suppressed both during and after the treatment period; *F. tularensis* agglutinins either did not appear or developed only in low titer.

TETRACYCLINE THERAPY

The initial objective in the therapy of acute tularemia is the rapid relief of clinical manifestations, an objective readily accomplished with bacteriostatic drugs (see above). Thereafter, the problem is the same as that in prophylaxis—the suppression of multiplication for sufficient time for host mechanisms to eradicate the microorganisms. The major difference, then, in the two situations is the extent of microbial multiplication, and supposedly the degree of stimulation of defense mechanisms, prior to initiation of treatment. Therefore, after control of clinical illness, therapeutic regimens similar to those found effective in prophylaxis should result in a negligible relapse rate, even if therapy is instituted early in the course of disease.

Volunteers exposed to 25,000 airborne *F. tularensis* (see above) became acutely ill after a mean incubation period of 3 days (range of 2 to 7 days). The onset of illness was gradual in 15% of subjects, and a biphasic course was occasionally observed. Treatment was instituted early, within 48 hr of initial signs of illness in 85% of the men, and in no case later than the 5th day after initial signs. Large doses of tetracycline were administered during the first 24 hr, i.e. 1 g every 6 hr, to insure high initial blood levels, and the daily maintenance quantity was administered thereafter in four equal doses.

During the initial phases of evaluation of tetracycline therapy, intermittent treatment schedules were examined. Therapy consisting of three five-day courses of tetracycline (0.5 g every 6 hr) separated by 3 days without drug was efficacious (Fig. 4); the patients responded rapidly and remained well thereafter. These results led
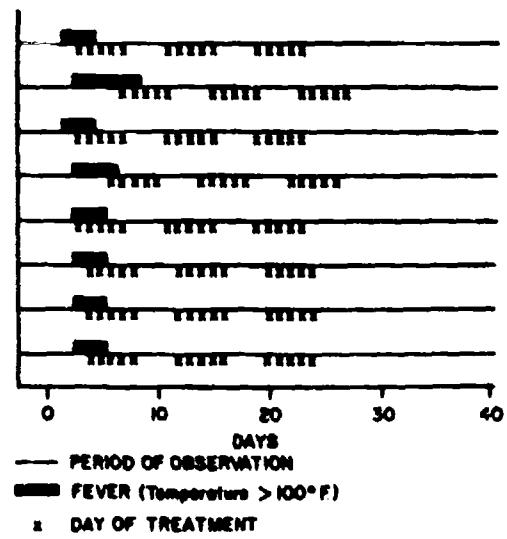
- PERIOD OF OBSERVATION
- FEVER (Temperature > 100° F)
- DAY OF TREATMENT

FIG. 4. *Interrupted tetracycline therapy of human airborne tularemia.*

PERIOD OF OBSERVATION
FEVER (Temperature >100°F.)
MINOR SYMPTOMS WITHOUT FEVER
r DAY OF TREATMENT
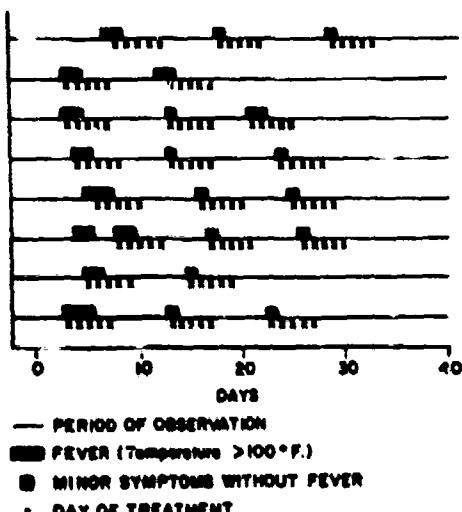
FIG. 5. *Interrupted tetracycline therapy of human airborne tularemia.*

to an attempt to reduce the number of treatment periods. After the initial 5 days of therapy, no additional drug was given to another group of volunteers until ordered by the ward physician, who was instructed to institute treatment at the first sign, no matter how equivocal, of a recurrence. The results were unsatisfactory (Fig. 5). In most instances, the recurrences were so rapid in onset that the men were disabled by the time treatment was effectively instituted; i.e., the time between recognition of possible recurrence and achievement of effective levels of drug in the patient exceeded the time required for the illness to progress from well-being to disability. Moreover, three courses of treatment were administered in 6 of the 8 subjects. It seemed, therefore, that there was little likelihood of developing effective interrupted treatment schedules either employing substantially less drug or of shorter duration than that originally evaluated. Later studies (see below) indicated that continuous tetracycline therapy with similar quantities of drug was equally effective; interrupted therapy, therefore, did not offer any advantage over the simpler continuous treatment schedule.

Prompt clinical improvement was achieved with the continuous therapeutic regimens listed in Table 4. Treatment with 22 g of tetracycline in 10 days resulted in a high incidence of relapse. The same daily dose continued through 15 days was not, however, followed by relapse in any of the 20 patients, 12 infected with the SCHU-S4 strain and 8 infected with the SCHU-S5 strain. The SCHU-S5 strain differs from SCHU-S4 strain

only in resistance to streptomycin, SCHU-S5 resisting more than 1.000 µg/ml. When the daily dose was halved, two of eight men had a relapse after 15 days of therapy.

As predicted, the simplest and most successful tetracycline regimen for prophylaxis was very similar to the best therapeutic regimen, i.e., 1 g of tetracycline twice daily for 14 days compared with 4 g of tetracycline the 1st day followed by 0.5 g four times daily for 14 additional days. From a practical standpoint, it would be desirable to have a single schedule of tetracycline administration for both prophylaxis and therapy of airborne tularemia. Therefore, six volunteers with acute illness were treated exactly according to the schedule found successful for prophylaxis; all recovered rapidly and remained well. Thus, a simple schedule of tetracycline treatment was effective in both prophylaxis and therapy of human airborne tularemia; that schedule was 1 g of tetracycline twice daily for 14 days. Because this treatment schedule was suitable for infections induced by exposure to a large number of organisms, the regimen should be satisfactory over the entire range of exposure encountered either in nature or in laboratory accident.

OTHER ANTIBIOTICS

For infection with streptomycin-sensitive *F. tularensis*, the clinician has a choice of effective antibiotics, particularly streptomycin and tetracycline. In cases of infection by streptomycin-resistant organisms, effective alternatives to tetracycline are needed. Therefore, a number of antibiotics active against *F. tularensis* SCHU-S5 (streptomycin-resistant) in vitro have been evaluated in the therapy of airborne infection of monkeys with the SCHU-S5 strain (Table 5). The inhaled dose was 10,000 organisms; 12 control monkeys became ill within 72 hr and died between the 7th and 15th day after exposure. Therapy was started early, i.e., after 12 hr with a

TABLE 4. *Tetracycline therapy of human airborne tularemia*

Daily dose[a]	Days of therapy	No. of subjects	No. with relapse
1			
2	10	11	5
2	15	20[b]	0
1	15	8	2

[a] All men received 4 g of drug the 1st day of therapy. Daily dose was given at 6-hr intervals.
[b] Twelve men infected with the SCHU-S4 strain and eight with the SCHU-S5 strain of *Francisella tularensis*.

TABLE 5. *Antibiotic therapy of Macaca mulatta infected with Francisella tularensis SCHU S5*

Antibiotic	Daily dose[a]	No. of monkeys	No. with slow re[b]	No. with
	mg			
Tetracycline	225	8	1	8
Kanamycin	90	8	2	0
Novobiocin	135	6	1	5
Gentamicin	9	8	8	3

[a] Divided into three doses. Therapy was continued for 7 days or until the animal was afebrile for 72 hr whichever was longer.

[b] An animal was classified as having a slow response if more than 72 hr of treatment were required before it became afebrile.

temperature >40 C or a single temperature of 41 C or greater. Tetracycline treatment resulted in rapid response, but, as expected, relapses followed this short course (see Table 5). Kanamycin, which was bactericidal in vitro, was bactericidal in vivo as well, and effected cure, albeit the initial response was somewhat slow in two monkeys. Novobiocin (Eigelsbach, Herring, and Halstead, Bacteriol. Proc., p. 69, 1957) gave results similar to those obtained with tetracycline. Although gentamicin was quite active against *F. tularensis* SCHU-S5 in vitro, therapy with it was disappointing. All monkeys responded, but only slowly; three of the eight had a relapse.

These results suggest that novobiocin may be employed in the therapy of human tularemia, but that prolonged courses, such as those found necessary with other bacteriostatic drugs, are likely to be necessary if therapy is to be completely successful. Although the bactericidal drug kanamycin was highly effective, its toxicity is such that it cannot be recommended for primary treatment. It may, however, be of value as a "backstop" in chronic, recurring infections and as an alternative to the broad-spectrum drugs in the management of infections by streptomycin-resistant organisms.

SUMMARY

Unlike streptomycin, tetracyline and the other broad-spectrum antibiotics do not kill susceptible *F. tularensis* in vitro or in vivo. The broad-spectrum drugs owe their effectiveness to their bacteriostatic action; they check multiplication of the invading organisms until host defense mechanisms can eliminate the bacteria. Elimination of *F. tularensis* within cells proceeds slowly, and organisms may persist for many days in man (and many weeks in monkeys) during tetracycline treatment. The results of the present studies of

tetracycline treatment indicate that infection with *F. tularensis* can be eradicated through bacteriostatic antibiotic therapy provided (i) that the antibiotic is administered in amounts sufficient to obtain continuous suppression of growth of intracellular organisms, and (ii) that the regimen is maintained for a sufficient period of time. These objectives have been met by a regimen of 2 g of tetracycline daily for 14 days. This regimen may be employed both for prophylaxis and for therapy of human airborne tularemia.

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Discussion

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The elegant work of Dr. Sawyer and his colleagues presents data which are of considerable importance, not only to those dealing with tularemia and related diseases, but also to many investigators who are interested in the general principles of antibiotic therapy and prophylaxis. This discussion is based on a general perspective, with emphasis on the prophylactic aspects.

All chemotherapeutic activity must be viewed in terms of the therapeutic ratio concept. Since there is rarely an assurance in natural situations that any given individual upon exposure will develop a clinical illness, the therapeutic ratio in the prophylactic situation has to be expressed in terms of group risk, group results, and group toxicity. Thus, if only one-half of an exposed population is destined to become ill and the prophylactic regimen gives no better end results than the treatment of half the group of subjects who actually become ill, the toxicity is doubled for the group as a whole and the results are no better than those of therapy; hence, the therapeutic ratio is less favorable. Since the accentua-

tion of toxicity is the most marked effect of prophylaxis, prophylaxis is usually attempted with the least toxic drugs or with a reduced dose. In addition, because it is often not possible to determine with accuracy the expected infection rate in the natural situation, it has been more difficult to measure prophylactic than therapeutic benefits. Clearly, the animal and volunteer studies of Sawyer meet the problem of evaluation well and thus provide important insight. Unfortunately, even the results of treatment of a random sample of an exposed population in a semiclosed situation, when the infection rate is unpredictable, may be difficult to interpret, since the treatment of some members of the group may influence the infection rate among the untreated.

The most important principle illustrated by these data is the primary importance of the host defense mechanisms. The superior results in man as compared with monkeys correlates well with the higher spontaneous recovery rates among the former. It is possible that much of what has been demonstrated is interpretable in terms of the

natural history of the disease and the defense mechanisms. That the agglutinins which were demonstrated were not capable of terminating the disease, per se, appears clear from the fact that the titers appeared early in those animals in which the prophylactic regimen failed to suppress symptoms even after the response had begun. On the other hand, in animals, given the 42-day course of treatment, which remained well thereafter, antibodies were not demonstrated. Measurement of a single type of antibody does not, however, preclude the presence or absence of others. A difference in the effectiveness of antibody as an anti-infectious agent may be part of the explanation for the differences between the success of the intermittent regimens in the rickettsioses in contrast to its failure here. The studies on vaccines, including those reported at this conference, do not lend much support to this hypothesis. Another factor accounting for the difference may be the relatively short incubation period of this disease; hence symptoms reappear in the brief drug-free interval, whereas the longer period in rickettsial disease may place the second course within the relapse incubation time. It would appear unlikely that intracellular parasitism accounts for the difference, since both organisms are intracellular and tetracycline, apparently at least, can influence them there. Perhaps extracellular phases may be of importance.

With respect to the type of stimulation of the defense mechanism, the attenuated nature of the post treatment relapses is of interest. This appears to be evidence of a partial stimulation of host immunity. It is conceivable, however, that in effect there has been a reduction in the surviving inoculum, so that the infection observed is of the same type as would have resulted from a sublethal dose without drug administration.

This study, of course, is immediately applicable to the disease studied, but is also probably a guide to other infections in which organisms survive within host cells. Perhaps the most important disease is tuberculosis. Can one reconcile with the data of Sawyer the results in the field trials of isoniazid prophylaxis in the person who has recently begun to react to tuberculin? In view of the fact that in tuberculosis the expected symptomatic infection rate is well below that seen in these volunteer infections, one might attribute more to the host defenses than to success of the regimen. That some break-throughs do occur even when there is good evidence that the drug is being taken, but that many more occur when it is stopped prematurely, suggests that the situation is similar, even though the rates are proportionally much lower. This similarity is observed in spite of the fact that isoniazid has equally favorable distribution characteristics and is, in addition, more bactericidal than the tetracyclines used in these experiments. The question may well be raised as to what the intracellular location offers in the way of protection to the pathogens. In the tularemia model, at least, there is no reason to suspect that tetracycline becomes ineffective by causing the production of wall-less forms, but a relatively dormant state might well persist, sheltered intracellularly from host destruction until after the drug disassociates from the anabolic mechanisms and multiplication of the organisms begins again. If this is so, one might expect a difference between prophylaxis preceding inoculation and treatment during the incubation period. Effective treatment of tuberculin-negative children who have contact with infectious persons suggests that this may be so. Dr. Sawyer's model could give some guidance on this point, which is not well established by the field trials. By extending the number treated to a still lower risk group, the therapeutic ratio is made less favorable, so more precise data would be appropriate.

Of interest is the fact that tetracycline is bacteriostatic, and the results are not as satisfactory as those with kanamycin, which is bactericidal. Several of the most successful prophylactic regimens have been the bacteriostatic sulfonamides used in relatively small doses against susceptible strains of meningococci and group A streptococci. Tetracycline prophylaxis also appears to be effective against the latter, but less so against the former. These unexpectedly good results may be related to inoculum size or to the extracellular nature of these infections, or both. In the case of the meningococcus, the sulfonamides work both pre- and postinoculation, even in quite small doses for short periods. The least explained exception to the rule of relatively incomplete activity of bacteriostatic drugs is the very low relapse rate among patients with *Haemophilus influenzae* meningitis, even though there is little reason to suspect great recovery powers on the part of the host, as judged by the natural history of the untreated disease. Similarly, we observed tetracycline to be quite successful in preventing *Haemophilus* infection of the upper airways in patients on a rheumatic fever ward. All of these results suggest a marked difference in those situations in which the defense mechanisms rely heavily on a pyogenic response and those in which they do not.

In many practical applications of prophylaxis, the results are greatly affected by the fact that any one of a number of strains of the same or of different species may give similar difficulty. When

superinfecting strains are more drug-resistant, prophylaxis may even be detrimental.

Since in the "virgin" community the susceptible forms almost always predominate over the resistant types, they must have a survival advantage of some not yet defined sort. Theoretically, such an advantage may be great or small. If it were great, it is likely that a much larger proportion of a population would have to be treated and thus denied to the susceptible strains, in order for the shift to occur toward a resistant population among the untreated. In such a case, the level of use required to initiate such a shift would be a good index of the potential for a drug to cause harm upon mass usage. Our recent experiences in a closed community suggest that the breeding of resistant, aerobic, gram-negative rods of the coliform type and of staphylococci starts when chloramphenicol is used simultaneously in about 5% of the population and increases linearly with usage up to the 30% level. These resistant populations among untreated patients within 2 weeks reach a level as high as 50% of the positive untreated carriers. With *Escherichia coli*, the spread pattern of resistant serotypes is clearly demonstrable as early as 36 hr after the drug has been introduced to subjects on the ward. Careful analysis of the serotypes and phage types indicates that chloramphenicol and tetracycline are good prophylactic agents in these infections when the strains are susceptible, but the effect is masked by the propagation of resistant populations. Against susceptible strains, they are at least as effective as bactericidal agents are against the streptococci.

To meet this problem, there have been several approaches. One is to treat quite briefly at the time of maximal exposure, for example, during surgery. Another consists of using the drug locally in high concentrations often in such a way that resistant strains might not be spread readily, since the area being treated is not one of the primary portals of spread. This type of prophylaxis is best illustrated by the use of antibiotics within the urinary bladder and of sulfamyelon on burns. The situation is less clear for the use of protective nasal ointments, although there have been some successes. Of interest to this conference might be the reinvestigation of aerosol prophylaxis and treatment. Previous poor results might well have been conditioned by improper sterilization of the equipment and the results falsely attributed to failure of the method.

One might predict that, for systemic infections with specific highly virulent strains, systemic prophylaxis will continue to be developed within the limitations of a favorable group therapeutic index. For those situations in which there is a potential for a resistant organism, present among the varied and mixed flora, to become dominant and, in addition, in which there is often a markedly impaired host resistance, a further trial of local regimens will probably be made.

Physical and Chemical Stresses of Aerosolization

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INTRODUCTION

Many investigators are concerned with the study of the stresses of aerosolization. As stated by Madin in the foreword to the First International Symposium on Aerobiology (15), they may be said to be engaged in studies oriented toward finding the most tolerable conditions under which bacteria may live in the airborne state and be infective. An entire session of the Berkeley Symposium entitled Survival and Viability was devoted to describing studies dealing with the stress of dehydration and its effect upon the viability of microorganisms. This stress was shown to be of major and primary importance in the study of airborne infection.

Dunklin and Puck (6) suggested that the sensitivity of microorganisms increases at a critical degree of dehydration. They based their suggestion on observations on the variation of the death rate of airborne bacteria with variation in relative humidity (RH). Webb (24) attributed the lethal effects of dehydration in the aerosol to a physical change in the structure of an essential macromolecule when water bound to this molecule is removed. Later, Webb (25, 26) stated that the bound water molecules appear to occupy strategic positions in the molecule, and only certain chemicals capable of forming hydrogen bonds of the correct type can replace them and maintain the biological integrity of the macromolecule. Zimmerman (29), reporting studies on both freeze-drying and aerosolization, proposed that nonpermeable sugars counteract the stress of aerosolization through a plasmolytic dehydration of the organism. Conversely, freely penetrating sugars are required to minimize the effects of the stress of freeze-drying.

Monk and McCaffrey (16) showed that the death rate of rehydrated *Serratia marcescens* is maximal at a water content of 33%, but stated that the effects of oxygen on the death rate had not been determined (17). Heckley and Dimmick (9) remarked that a study of lyophilized orga-

nisms has a place in aerobiology because freeze-dried organisms are similar to airborne cells in that they are essentially naked and in direct contact with the atmosphere. Earlier, Davis and Bateman (2, 3) had investigated the killing of freeze-dried *Escherichia coli*, *Micrococcus lysodeikticus*, and *S. marcescens* upon exposure to water vapor in vacuo, and had made a qualitative correlation between susceptibility to injury and surviving oxidative metabolism of the cell types. The assumption common to most of this work has been that a change in cellular water (dehydration or rehydration) causes the death of the cell by the physical disruption of vital structures, by concentration of toxic chemical material within the cell, or by creating an imbalance in metabolic activity.

EFFECT OF OXYGEN

Rogers (19) was one of the first investigators to recognize the lethal effects of oxygen on lyophilized organisms. Naylor and Smith (18) have reported results in agreement with those of Rogers. These investigators reported that survival is highest for organisms stored under vacuum and lowest for those stored in air or oxygen. Atmospheres of nitrogen, hydrogen, and carbon dioxide yield intermediate results. Scott (20) reported that the effect of the atmosphere upon the survival of dried bacteria depends upon the nature of the suspending medium and its moisture context. Recently Lion and Bergmann (12, 13) listed numerous substances that protect lyophilized *E. coli* against the lethal effects of oxygen. Lion (14) suggested that a prerequisite for effective protection against oxygen in the dry state is the accumulation of the solute around the bacteria, which he assumed to occur during lyophilization. Benedict et al. (1) reported that atmospheric oxygen kills 95% of dried *S. marcescens* in 10 min, that certain reducing agents prevent the action of the oxygen, and that humidity seems to play no role in the phenom-
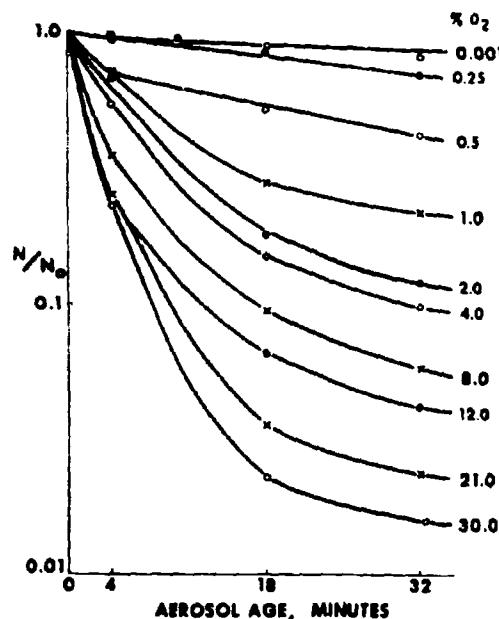
FIG. 1. *Survival of aerosolized Serratia marcescens after contact with various concentrations of O_3 at 40% RH, 25 C. Suspensions contain washed cells in water with 20×10^9 viable S. marcescens cells per milliliter and 2.0×10^9 viable Bacillus subtilis spores per milliliter. N/N_o = ratio between viable S. marcescens and B. subtilis spores collected from the same aerosols, corrected for differences in control viable-cell counts: P_o = atmospheric pressure, P = partial pressure of O_3. Results of one typical set of trials.*
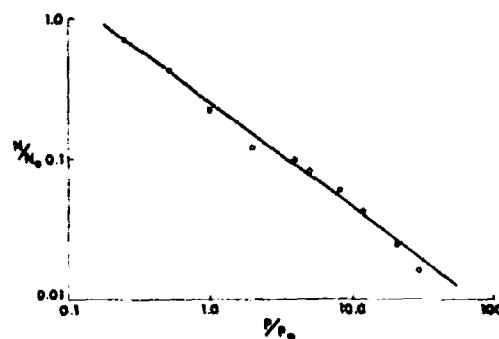
FIG. 2. *Survival of aerosolized washed Serratia marcescens after 32 min versus O_3 concentration. Same symbols as for Fig. 1.*

FIG. 3. *Survival of washed Serratia marcescens in nitrogen with $5\% O_3$ added at times indicated by arrows. Same symbols as for Fig. 1.*

TABLE 1. *Survival of washed and unwashed Serratia marcescens aerosolized into air or N_3[a]*

Aerosol age	N/N_o[b]			
	Air		N_3	
	Unwashed	Washed	Unwashed	Washed
min				
4	0.009	0.230	1.0	1.0
18	0.0007	0.040	0.92	0.95
32	0.0001	0.024	0.90	0.90
150	—[c]	0.007	0.72	0.70
300	—[c]	0.004	0.70	0.69

[a] Suspensions contained 20×10^9 S. *marcescens* cells per milliliter and 2×10^9 *Bacillus subtilis* var. *niger* spores per milliliter. All aerosols generated at 40% RH, 25 C.
[b] Ratio of S. *marcescens* to B. *subtilis* spores in the same aerosol, corrected for differences in control counts.
[c] Too low to assay.

enon. Wagman (23), however, demonstrated a marked dependence of survival upon residual moisture in studies of circulating-gas (air) freeze-drying of water-washed S. *marcescens* and E. *coli*.

Studies of the effects of ascorbic acid on aerosolized S. *marcescens* in our laboratory suggested that interaction between the cells and atmospheric oxygen may contribute to the death

TABLE 2. *Effects of various compounds on survival of aerosolized Serratia marcescens*[a]

Compound	Concn	N/N_0[b]		
		4 min[c]	18 min	32 min
	moles/liter			
None (air, 97% RH)	—	1.07	1.03	1.00
None (air, 40% RH)	—	0.23	0.04	0.02
None (N₂)	—	1.00	0.95	0.90
MnSO₄	5×10^{-2}	0.77	0.51	0.48
MnCl₂	3×10^{-3}	0.47	0.37	0.34
Mn(NO₃)₂	2×10^{-4}	0.41	0.35	0.19
MgSO₄	2×10^{-2}	0.28	0.11	0.07
CoCl₂	2×10^{-4}[d]	0.52	0.22	0.13
NaCl	8×10^{-2}	0.06	0.03	0.01
CuSO₄	2.5×10^{-4}[d]	0.20	0.04	0.03
Glycerol	1×10^{-2}	0.47	0.28	0.18
Thiourea	1×10^{-2}	0.44	0.25	0.21
Cysteine-HCl[e]	5×10^{-2}	0.26	0.10	0.06
N-ethylmaleimide (air)	1×10^{-4}[d]	0.20	0.07	0.05
N-ethylmaleimide (N₂)	1×10^{-4}[d]	0.75	0.44	0.40

[a] Suspensions contained 20×10^6 S. *marcescens* cells per milliliter and 2×10^6 *Bacillus subtilis* var. *niger* spores per milliliter. All aerosols were generated in air at 40% RH, 25 C, except as noted.

[b] Ratio of S. *marcescens* to B. *subtilis* spores in the same aerosol, corrected for differences in control counts.

[c] Aerosol age.

[d] Higher concentrations were toxic to control suspensions.

[e] Brought to pH 7.0 with NaOH.

of cells. Preliminary experiments indicated that, when S. *marcescens* is aerosolized into air diluted with nitrogen, the death rate increases with oxygen concentration.

Work was undertaken to test the possibility that drying sensitizes organisms to lethal effects of oxygen but in itself is not the direct cause of death of the microorganisms. Hess (10) tested the effects of oxygen on aerosolized S. *marcescens*, and Dewald (5) has studied the kinetics of the effects of oxygen on lyophilized S. *marcescens*. It is appropriate to describe the results of their studies in some detail.

AEROSOL EXPERIMENT

Hess (10) aerosolized water suspensions of S. *marcescens* (ATCC strain 14041) in a rotating drum of 86.6-liter capacity revolving at 5 rev/min (8). *Bacillus subtilis* spores were used as a tracer to indicate maximal viable-cell recovery. Ratios of viable S. *marcescens* and B. *subtilis* spores from aerosols stored in various concentrations of oxygen are shown in Fig. 1. Maximal survival of the S. *marcescens* occurred at the minimal oxygen concentration attained, and at that point was nearly equivalent to spore survival. As the oxygen concentration was increased, loss in viability increased so that $\log N/N_0 = k \log P/P_0$

+ C (Fig. 2). All aerosols were generated from thoroughly washed cells free from added solutes. The tests were performed at 40% RH because aerosols of this organism routinely yield minimal survival in air at this humidity.

Lethal effects were observed when O₂ was added to aerosols originally disseminated into N₂ (Fig. 3). Although only 30% loss in viability occurred during 5 hr in nitrogen (Table 1), the addition of 5% oxygen resulted in at least 80% loss in viability within 30 min. This effect was noticed at oxygen concentrations as low as 0.25%, and became greater as oxygen concentration increased. The addition of 5% oxygen after aerosols had been stored in nitrogen for up to 40 min was selected as an arbitrary example of this system.

Unwashed, unstarved S. *marcescens* cells were more sensitive to storage as aerosols in air (Table 1) and respired 5 to 10 times faster than washed shaken cells. Stability in nitrogen was unimpaired, however, indicating a relationship between respiration and sensitivity to oxygen.

Effects of several additives on the stability of aerosolized S. *marcescens* are shown in Table 2. These compounds were selected because of their demonstrated influence on the stability of enzymes in organisms exposed to oxygen in other systems. The concentration of each compound inducing
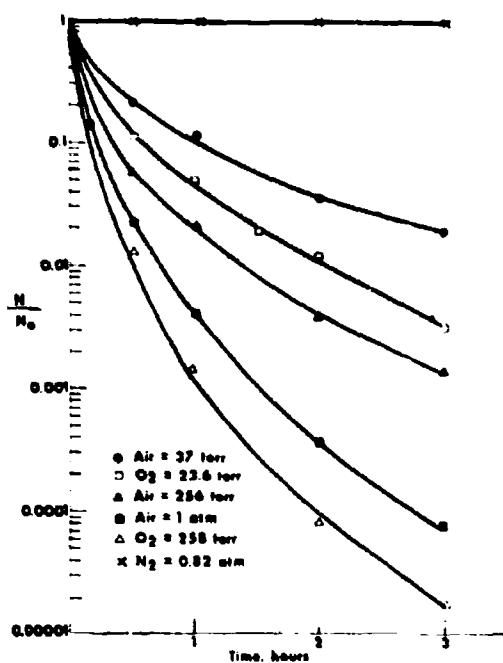
FIG. 4. *Semi-log plot of survival versus time after exposure of lyophilized Serratia marcescens to various pressures of oxygen dry air, or purified nitrogen. N_0 and N are the number of viable organisms before and after the exposure, respectively.*

FIG. 5. *Semi-log plot of survival versus oxygen pressure after 0.5- (line A) and 1-hr (line B) exposures to oxygen or partial pressures of oxygen in dry air; N_0 and N are the number of viable organisms before and after exposure, respectively. Open circles and squares, pure oxygen; closed circles and squares, partial pressure of oxygen in dry air.*

optimal stability in the aerosol was determined empirically.

Experiments were performed to determine the sensitivity of completely hydrated *S. marcescens* to oxygen. Completely hydrated organisms were insensitive to oxygen at pressures up to 100 psi for 4 hr, and no viability loss occurred in aerosols of washed cells in air at 97% RH.

EXPOSURE OF LYOPHILIZED ORGANISMS

Dewald (4) has developed a high-vacuum method of lyophilization of *S. marcescens* that yields 45 to 70% survival of the parent suspension; he has used this material to study effects of exposure to oxygen and air (5).

Data on survival versus time, obtained by exposing lyophilized *S. marcescens* at various pressures of oxygen, dry air, and nitrogen, indicate a dependence upon partial pressure of oxygen similar to that observed in the aerosolization studies of Hess (Fig. 4). No loss in viability could be detected when the dried organisms were held under vacuum for periods up to 3 hr at pressures less than 10^{-5} torr. The dependence of viability upon the partial pressure of oxygen

after 0.5- and 1-hr exposures at 25 C was demonstrated (Fig. 5). Another representation of the inactivation curves (Fig. 6) shows that the survival data can be linearized by plotting log N/N_0 versus (time)$^{1/2}$, leading to a rate expression, $-\ln N/N_0 = Kt^{1/2}$, where K is a pseudo rate constant that in turn can be related to the oxygen concentration by $K = k [O_2]^n$ or $\log_{10} K = n \log_{10} [O_2] + \log_{10} k$.

The pseudo rate constants, K, for all the inactivation data determined by least-squares fit and related k values are given in Table 3. The log of the pseudo rate constant versus log oxygen concentration is given in Fig. 7, and leads to the following expression for the inactivation of the freeze-dried material by oxygen:

$$-\ln N/N_0 = K[O_2]^{1/2}t^{1/2}$$

where $k = 276 \pm 36$ moles $^{-1/2}$ cc$^{1/2}$ hr$^{-1/2}$ at 25 C.

The Arrhenius function was determined for the dried organisms exposed to dry air at atmospheric pressure for 1 hr at temperatures ranging from
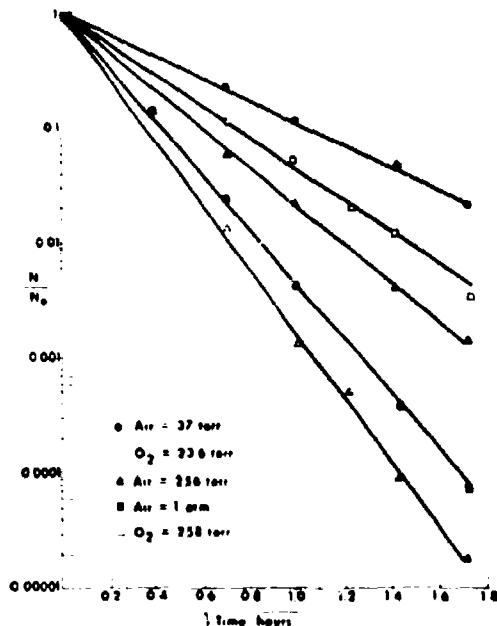
FIG. 6. *Semi-log plot of survival versus (time)$^{1/2}$ after exposure of lyophilized Serratia marcescens to various pressures of oxygen or dry air. N_0 and N are the number of viable organisms before and after the exposure, respectively.*

TABLE 3. *Kinetic data for the inactivation of Serratia marcescens by oxygen*

Oxygen pressure (torr)	Oxygen concn^a (10^{-7} mole/cc)	K pseudo ($hr^{-1/2}$)	k (moles$^{-1/2}$ cc$^{1/2}$ hr$^{-1/2}$)
258	139	6.51	271
172	92.5	5.50	261
160^b	86.1	5.17	252
159^b	85.5	6.13	300
121	65.0	5.27	283
110^b	59.2	4.79	275
71.6	38.5	4.15	264
53.7^b	28.9	3.85	271
37.7	20.3	4.28	337
30.8^b	16.6	3.02	258
23.6	12.7	3.31	306
11.0	5.92	2.11	251
8.0	4.30	1.85	245
7.8^b	4.20	2.29	306
5.5	2.96	1.78	267

^{a} Calculated by use of the ideal gas law.
^{b} Dry air used as source of oxygen.

-78 to 40 C. The results, plotted as Fig. 8, yield, by least-squares fit:

$$k = 10^{5.94 \pm 0.43} \exp \left[(-430 \pm 26) \text{ cal}/RT \right]$$
$$\text{moles}^{-1/2} \text{ cc}^{1/2} \text{ hr}^{-1/2}$$
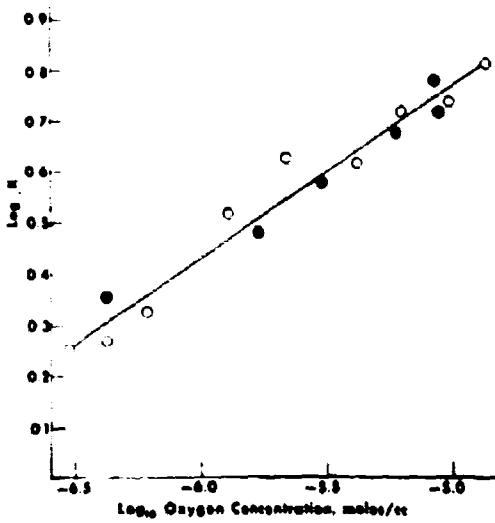
FIG. 7. *Log$_{10}$ of the pseudo rate constant, K, versus log$_{10}$ of the oxygen concentration. Open circles, pure oxygen; closed circles, dry air.*
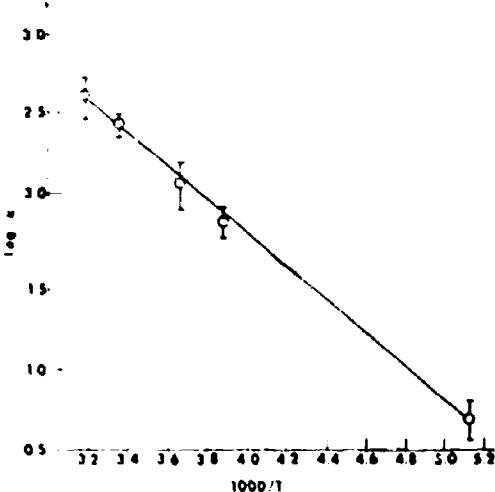
FIG. 8. *Arrhenius plot for the inactivation of Serratia marcescens by oxygen. On the abscissa, T is in degrees Kelvin.*

DISCUSSION AND OUTLOOK

The results here can be compared with those of aerosolization inactivation (Fig. 9). There appears to be no pronounced difference in the degree of inactivation after 0.5 hr as a function of the partial pressure in the two systems. The data of Hess were obtained at 40% RH; 0% RH was used in the lyophilization-exposure studies;
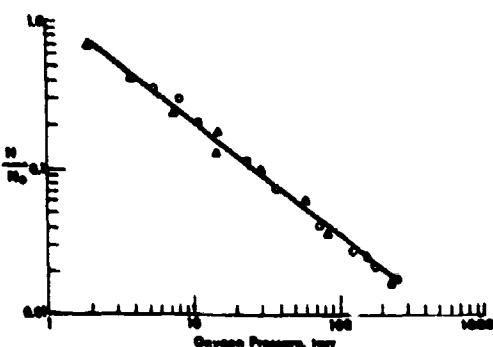
FIG. 9. *Log-log plot of survival of Serratia marcescens versus partial pressure of oxygen. N₀ and N are the number of viable organisms before and after the stress. Open circles, this work, lyophilized organisms after 0.5-hr exposure; closed triangles, aerosolized organisms after 32 min.*

hence, direct comparison is not completely valid. Additional and preliminary studies indicate that the survival of washed, lyophilized *S. marcescens* exposed to oxygen in humidified air is essentially independent of RH between 0 and 85%, whereas the survival of the organisms exposed under the same conditions, but lyophilized from suspensions containing 0.05% NaCl, showed a marked dependence on RH. The comparison does indicate a remarkable similarity in degree of inactivation in the two systems. Although the mechanism resulting in death is not known, the data require a consideration of oxygen effects in explaining the stress of dehydration. Hess (10) has made a comparison with other systems, and he points out similarities with studies by Hollaender, Stapleton, and Martin (11) and Tallentire (21, 22) on the lethal effects of oxygen on irradiated organisms. Further, he recognized the hypothesis of Gerschman et al. (7) concerning a common mechanism between oxygen poisoning and the initial effects of X irradiation in biological systems, and he proposed that O₂ poisoning and death of airborne bacteria may be similarly related. Webb (27, 28) examined the effects of ultraviolet light and X rays upon air-dried microorganisms, and proposed that, in aerosols below 80% RH, death due to toxic chemicals, irradiation, or desiccation alone is the direct result of removal or reorientation of bound water.

It is clear that the stress of dehydration and the events that are initiated by application of this stress remain to be adequately defined. A complete description of the behavior of microorganisms and their reactions to the environment is required to understand airborne organisms and the complexities of airborne infection.

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Discussion

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The experiments on the disinfection by oxygen
of bacteria dried either by aerosolization or by
lyophilization presented by Mr. Zentner are
delightfully clean and precise, and are adequate
for some kinetic studies. This fact relieves us of
the relatively unrewarding task of discussing the
experimental techniques, accuracy of the data,
and validity of the conclusions. It makes it
possible to attempt to place the conclusions in
broader perspective, to attempt to interpret them,
and to examine whether they suggest or point the
way to further investigation.

It is perhaps surprising at first glance that
oxygen is toxic to *Serratia marcescens* under the
experimental conditions described. Mr. Zentner
has, I think correctly, related this implicitly
to dehydration of the bacteria. The observation
has been amply confirmed and is even intuitively
reasonable in terms of several hypothetical
mechanisms; for example, the concentration of
some toxic metabolite may increase at a critical
location because diffusion is limited by dehydra-
tion, or dehydration may distort some structure
and render it more susceptible to oxidation.

It is even more surprising that the biological
reactions observed follow so nicely the kinetic
laws described by the figures; in particular, there

is no threshold concentration for the toxicity of
oxygen (Fig. 2, 5, 7, and 9), and the data form a
straight line on the Arrhenius plot (Fig. 8)
over an extraordinarily wide range of temperature
(−78 to +40 C). Data on disinfection with a
variety of disinfectants commonly follow similar
kinetic laws (see, for example, F. H. Johnson, H.
Eyring, and M. J. Polissar, *The Kinetic Basis of
Molecular Biology*, p. 453 ff, John Wiley & Sons,
Inc., New York, 1954). The generality of this
type of biological data simply emphasizes the
importance of understanding why the biological
data follow these chemical laws.

The Arrhenius relation is usually derived from
a thermodynamic consideration of a chemical
reaction at equilibrium or from a statistical
mechanical consideration of reaction rates. In
the latter case, it is found necessary to introduce
the concept of an "activated state" in or near
equilibrium with the chemical reactants of the
system. Each molecule in an activated state has a
certain probability of decaying into its products
(or, by analogy, dying). The logical essence of the
reasoning leading to the Arrhenius relation, then,
is the existence of two states of the system at, or
nearly at, equilibrium with each other. One of
these states may be the "activated state" in which

the organism has a certain probability of dying. It places less strain on our credulity to accept a rather abstract interpretation of the experimental results such as this than to believe that each organism behaves like or is dependent upon a single molecule.

The very abstractness or generality of this interpretation of the kinetic data is a serious handicap, because it does not point the way toward a critical chemical substance or chemical or physical reaction, nor does it even say whether there are one or more "activated states" of the bacterium. In fact J. B. Bateman has gathered evidence that several different "activated states" may be involved in bacterial death due to dehydration. We may hope that the situation with regard to oxygen toxicity is not as complex, and hence as depressing, as that pictured by Bateman for dehydration disinfection. Continuing in this happy but somewhat illogical vein, we may say that the data presented by Zentner are consistent

with the existence of a single "activated state," and even suggest that the "activated state" is related to the accumulation of some intermediary metabolite closely involved with oxidative metabolism. In this way, the experiments presented by Zentner do suggest new experiments through which our understanding of oxygen toxicity might be improved, but they do not exclude wholly different types of possible mechanisms, such as free radical production, direct oxidation of critical material, or structural change in the bacterium induced by oxygen.

It is possible that a clever guess may permit an experiment which will elucidate the nature of an "activated state." Otherwise, before the disinfection of dried bacteria by oxygen or by other disinfectants can be understood and adequately described, a great number of experiments and measurements on many independent variables will be necessary, followed by a complex relaxation analysis of the kind outlined by Bateman.

Multistage Liquid Impinger

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INTRODUCTION

Instruments which collect viable airborne particles by inertial impact may be divided into two classes. Class I embraces instruments which project the viable particles straight onto the surface of a nutrient agar gel. Here they grow during incubation into one colony per viable *particle*, regardless of the size of the particle or the number of viable cells it contained. In class I are the slit-sampler, cascaded slit-sampler, sieve sampler, and the cascaded sieve or Andersen sampler, the features of all of which are conveniently summarized by Decker et al. (1). There is also the design of Lidwell (4). Class II samplers, with which we are concerned in this paper, project the particles into liquid, where they are broken up into their individual component cells. The liquid is serially diluted, plated out, and incubated to give a colony count and an estimation of the total viable *cells* in the original sample.

Typical of class II is the widely used Porton or capillary impinger (6, 14) in which a jet of air, accelerated to sonic velocity by suction in excess of 15 inches (38.1 cm) of mercury depression, impinges into liquid where small particles are collected with high efficiency. The sonic velocity jet serves also to limit the flow to a constant value.

The impinger is simple, cheap, convenient, easily sterilized, and, by the addition of a pre-impinger (5, 7), becomes an approximate simulant of the upper and lower parts of the respiratory system. Class II devices have the following features not possessed by those of class I. (i) They can cope with any high concentration of aerosol by virtue of the serial dilution process, whereas class I devices are easily overloaded. (ii) They permit counts of several different organisms from

the same sample, by means of differential counts or selective media. (iii) They are very convenient when nondecaying tracers are added as one component in an artificial aerosol. The tracer, such as a spore or radioactive element, allows the rate of decay of another component to be estimated, such as that of an organism under radiation stress. This estimation is independent of changes in the aerosol concentration. (iv) Virus aerosols can be estimated. (v) When the cascade system is used to give particle size discrimination and the aerosol of interest is highly skewed in size distribution, with many small particles and very few large ones, class I devices are subject to an error which in class II is negligible. This error arises from the shape of the cut-off curves for each stage of the system (see Fig. 3). The lower tail of the curve tends to flatten off along the abscissa, so that a few of the small particles will be caught on the large particle stage(s). In class I devices, these few particles will be recorded as large weighty particles which are in fact fictitious, but in class II their effect will be negligible in the final count of total cells per stage. Therefore, when the large particle count is of interest, it should be treated with reserve in class I devices, when there are many small particles present.

The special features of the impinger system are sometimes offset by its limitations. It is not very good with very dilute aerosols, in which it yields but a few cells widely dispersed in a large volume (10 to 20 ml) of liquid; the liquid, which is under low pressure, evaporates rather quickly and also tends to freeze in cool dry air; the violent impingement can kill delicate cells (6, 14).

It was deemed desirable to design a sampler which minimized the limitations of the Porton impinger and could be more widely used. The outcome is the subject of this paper. The new sampler has a higher sampling rate and gives a greater concentration of cells per unit volume of collecting fluid. The rates of evaporation and freezing are low and the impingement is gentle. The particle size discrimination is in three stages and is intended to simulate some of the finer details of the respiratory tract, where similar processes of inertial collection of particles operate. Also, the new sampler is more robust and more easily portable than the Porton impinger-preimpinger combination and is not subject to loss of collecting fluid through spilling as is the preimpinger. Finally, prolonged sampling periods do not result in as high a proportion of organism death as does the Porton impinger.

BASIC DESCRIPTION OF DESIGN

Standard Model

Drawings of the form of instrument most commonly used to date are shown in Fig. 1. A and B are sectional side elevations at right angles to each other in the directions I-I and II-II, respectively. The body is entirely of Pyrex glass, the outer parallel portions being blown from thick-walled tubing of 70-mm outer diameter. The sampler has three chambers or stages, 1, 2, and 3, in serial order vertically. The air inlet tube 4 has a smoothly curved entry to promote laminar flow, a flat ground lower end, and a bore of 15 mm. The straight tube 5, also with a smoothly curved bell-mouth, a flat ground lower end, and a bore of 10 mm is sealed into the flat floor of stage 1. This tube provides air-flow connection with the next stage. The tube 6, again with a smooth bell-mouth and bore of 10 mm, is sealed into the floor of stage 2. At its lower end, it bends and tapers smoothly and continuously to the nozzle 7, which has an internal diameter of 3.3 mm. The nozzle is close to the bottom of the annular well 8, formed as shown, and the axis of the nozzle lies in a plane tangential to the wall of the well and makes an angle of 45° to the vertical. Two circular discs, 9 and 10, made from coarse sintered glass 3 mm thick, are held about 1 mm above the flat floor of their respective chambers by the pairs of curved glass rods, 11 and 12, fused to the external walls of tubes 4 and 5, respectively. The discs 9 and 10 are twice the diameter of the bores of their respective tubes, 4 and 5, and are separated from the flat ends of these tubes by a distance equal to three-eighths of the bore. In use, the discs are constantly wetted by sampling fluid in each chamber. Access holes to each chamber are sealed by the rubber bungs, 13, 14, and 15. The lowest bung, 15, is fitted with a tube, 16, for connection to a suitable pump. This tube 16, which may be of any suitable material, projects into the center of the lower chamber and, as drawn in Fig. 1, may embody a flow-controlling critical orifice to give a constant throughput of 55 liters per min.

When operating the sampler in a cross-draft or wind, a hemicylindrical metal shield, 17, is clipped over the top so that the concave side faces upwind.

PRINCIPLES AND METHODS OF OPERATION

First Stage

When air is drawn through the instrument, air enters the intake tube, 4 (Fig. 1), and flows over the disc, 9, where some of the larger aerosol particles impact on the wet surface. To minimize
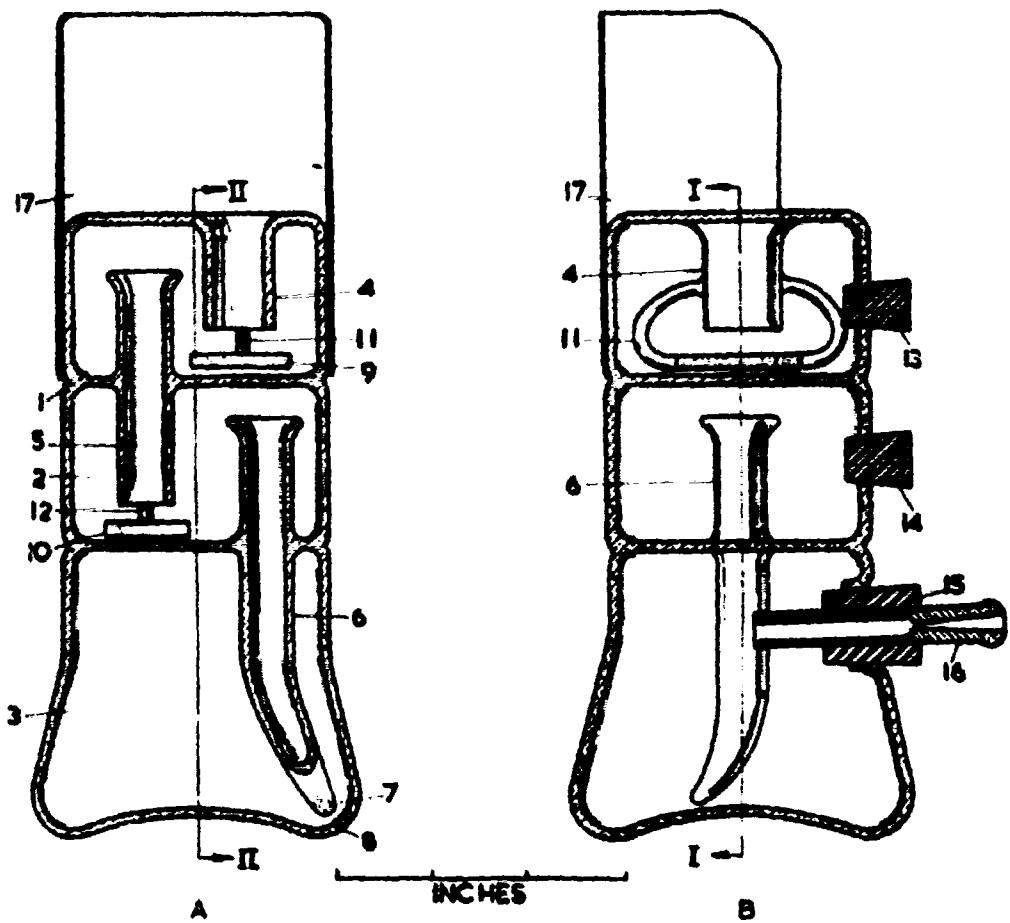
Fig. 1. *Sectional elevations of standard design.*

particle loss inside the intake tube, it should always be kept clean and dry. The ratio of 3:8 for (distance from tube to disc)/(tube bore) is that which is considered by Mitchell and Pilcher (11), and also by Mercer (9, 10), to give the steepest cut-off curve (Fig. 3), i.e., the sharpest particle-size cut-off. When the flow rate is 55 liters per min, 50% of bacterial particles 6 μ in diameter (d_{50}) with a specific gravity of about 1.5 will be trapped on the first sintered disc, and the proportion of particles of other sizes caught may be judged from Fig. 3. The rather high specific gravity is that measured for the dry bacterial clusters used in the calibration.

The air then flows outwards over the disc and liquid surface. It is particularly important that the sampler should be vertical and that the liquid should be nowhere higher than the upper surface of the sinters; otherwise, the outflowing film of air will encounter a standing liquid wave which will trap many particles intended for the next stage.

Second Stage

The same process of impaction and particle selection takes place at the second stage. With the smaller bore and higher velocity, the d_{50} is 3.3 μ, as shown in Fig. 3

Third Stage

The remaining particles now pass down the third tube, 6, and through the jet 7 (Fig. 1). It is important that tube 6 be smoothly tapered to the jet without any sharp bends; otherwise, particle loss on the inner walls will be excessive. For the same reason, it is important that tubes 4 and 5 should be quite smooth internally.

The tangential component of the jet direction
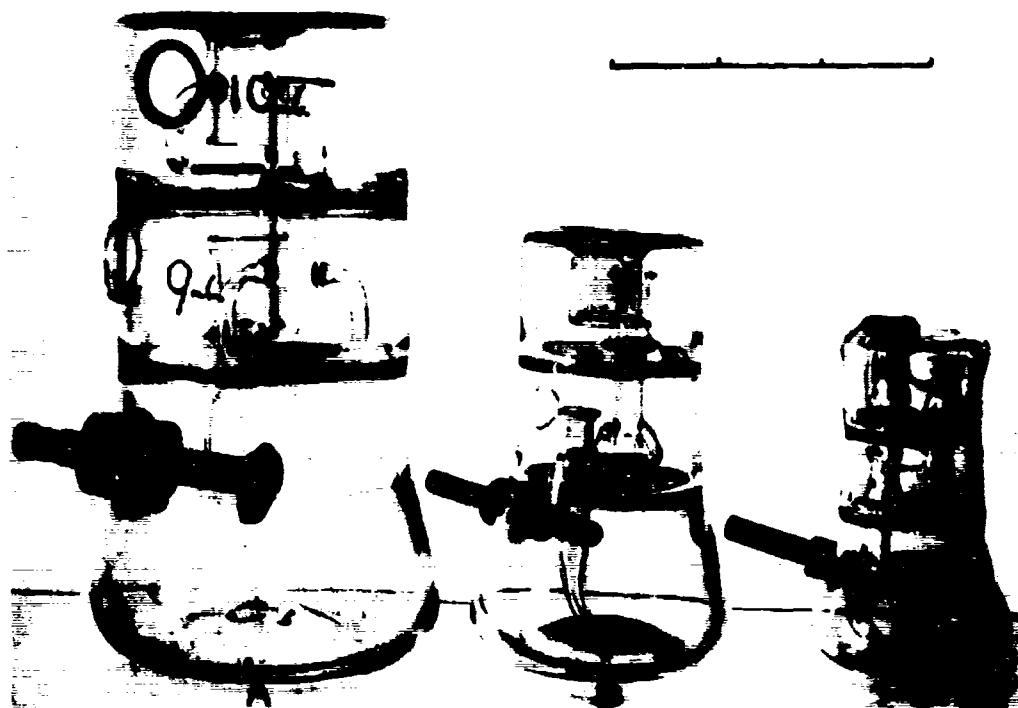
FIG. 2. *Three variations of basic design: (A) 50 liters per min model; (B) 20 liters per min model; (C) 10 liters per min model. Scale is in inches. Crown copyright reserved. Reproduced by permission of Her Majesty's Stationery Office.*

imparts a vigorous swirl to the liquid, which ensures that impingement is always on a wetted surface, provided that the liquid volume is not less than 5 ml. The diameter of the jet was determined on the basis of the performance of the "sub-critical" impinger described by May and Harper (6), and the idea of liquid swirl came from the Shipe sampler (14).

The jet gives no more than just sufficient velocity to ensure the capture of most single bacterial cells. Tests by G. J. Harper (*personal communication*) established that 80 to 90% of single cells of *Bacillus subtilis* and *Escherichia coli* were retained. The design has three advantages over the standard critical orifice impinger. First, the minimal violence of the impingement minimizes or perhaps eliminates kill of delicate cells. Second, splashing and frothing are minimized, so that a high air flow can be maintained through the compact third stage chamber without liquid loss by entrainment (note that the extract tube, Fig. 1 and 2, extracts air from the center of the chamber). Third, the critical orifice or other flow control system is downstream of the extract point, so that the pressure drop on the liquid

surface is only about 2 inches (5 cm) of mercury below ambient. In this way, both the rate of liquid evaporation and the possibility of freezing are very much reduced.

Liquid Loss by Splashing

Sometimes splashed liquid may collect on the roof of the third chamber and drip down on tube 16, whence it is entrained to waste through the tube. Such loss may be avoided by fitting the rubber drip ring seen in Fig. 2A.

Liquid Loss by Evaporation

If a long-period sample is taken, evaporation of the sampling fluid will occur. Evaporation is permissible to about 5 ml per stage, and the gap under the sintered discs will ensure circulation of liquid so that the disc remains moist. If evaporation threatens beyond this level, the lost liquid may be made up. With air at normal living conditions, topping-up may be necessary every 0.75 hr or so. About the same quantity will be lost from each of the three stages.

Sampling in Still Air

When the air to be sampled is calm or nearly so, as in a room, the sampler is used as it stands (Fig. 2). The smooth entrance, large diameter, shortness and smooth straight bore of the entry tube, 4, will ensure a high efficiency of collection of airborne particles.

Sampling in a Crosswind

In this case, the shield, 17, is fitted to stop cross-flow above the entry tube, thus approaching the still-air conditions. The system, which might be termed "stagnation-point sampling," is an entirely different concept from "isokinetic sampling" where air enters a knife-edged sampling tube with no change of speed and direction from that of the wind. Obviously, isokinetic sampling cannot be achieved from a horizontally moving air stream with impaction onto a horizontal liquid surface, as a 90° turn must intervene. In fact, true isokinetic conditions cannot be achieved outside a laminar-flow wind tunnel because of air turbulence. In stagnation-point sampling, the requirement for high intake efficiency would seem to be that the intake zone should be large compared with the particle's "stopping distance." The stopping distance is the distance of projection of the particle into still air from a given initial velocity, and varies as the square of the particle diameter and the velocity of projection. The validity of this concept is discussed below in the Calibration and Testing sections.

Sampling from a Tube

It may be required to withdraw aerosol samples from a chamber via a tube. In such a case, the connecting tube must never be inserted into the intake tube of the sampler by means of a bung, etc., as the jet effect from the narrow tube would completely alter the collection characteristics of the first stage. Ideally a 2.75-inch (7-cm) bore rubber hose would be pressed over the whole of the top of the sampler, maintaining the same wide bore to its source. If this is difficult, a tube at least as wide as the intake tube over the whole of its length should be used for the connection, with a wide metal flanged piece at its end. A sponge-rubber ring should be fitted under the flange, and the whole should be pressed firmly on top of the sampler.

Filling with Sampling Fluid

Fluid suitable for the organisms of interest is pipetted into each stage through the bung holes. The two upper stages are filled until the liquid surface is just below the upper surface of the sintered disc when the sampler is standing vertically. The upper surface of the disc will always be wet by capillarity, provided that the sinter is maintained in a grease-free condition by appropriate cleaning procedures. The standard model requires between 7 and 10 ml, varying from sampler to sampler, because of the vagaries of glass-blowing. It is convenient to mark indelibly by the side of each bung hole the volume required, as shown in Fig. 2A. Into the lower stage, 10 ml is pipetted.

Emptying

After use, the sampler may be shaken gently to mix the liquid, which is then withdrawn by a 10-ml calibrated pipette, to measure the volume. The liquid should be squirted a few times over the sintered disc to ensure removal of all organisms. This, and subsequent pipette mixing, must be done vigorously to ensure breakdown of clumps into single cells.

Sterilization and Cleaning

Before autoclaving or heat-sterilizing the sampler, the rubber bungs must be removed. When collecting fluid containing dissolved solids has been used, the sintered discs should be washed thoroughly with distilled water before heating, so that solids do not get baked into the pores. The sintered glass must always be kept chemically clean.

Flow-rate Control

Any system of flow control may be used, but the Hartshorn or venturi-shaped critical orifice (16 in Fig. 1) is perhaps the most convenient. These orifices constrict in a smooth curve to the throat, and then expand at an included angle of 4° over a length of about 1 inch (2.5 cm). As pointed out by Druett (2), this orifice geometry allows critical flow to commence at a very small depression, 4 to 5 inches (10.2 to 12.7 cm) of mercury compared with 15 inches (38.1 cm) for a conventional parallel-sided orifice. A substantial economy of pumping power is thus afforded. The addition of the 2-inch pressure drop across the sampler gives a safe minimum of only 7 inches of mercury depression required from the pump at the full flow rate of the sampler. Critical orifices are made slightly undersize and then reamed out until, in situ in the sampler, the desired throughput is achieved. In the present case, the initial bore is 2.5 mm for final adjustment to 55 liters per min. Systems of control not embodying a critical orifice are (i) a flowmeter with valve, downstream of the sampler; (ii) a compressed air or steam ejector operating at a constant pressure

(although these can only give a small pressure drop on their suction side, it is adequate for the 2 inches of Hg that this sampler requires); (iii) a sensitive pressure gauge just downstream of the sampler followed by a valved T-piece in the pump suction line. The valve is adjusted to bleed ambient air into the line until the depression across the sampler is that which in prior calibration gave the correct flow. The latter system makes the least demands of all on the pump but must be watched to maintain steady flow unless a sensitive pressure-regulated bleed valve is built into the T-piece.

Other Features of the Design

Wet-disc Collection Surfaces

The disc surfaces are moist, as are particle deposition areas in the respiratory system. This similarity is important in that the retention capability of a surface undoubtedly depends on its physical state as well as on the physical state of the surface of the particle.

Another valuable feature of the sintered discs is that their geometry, hence, impaction efficiency, is unaffected by the gradual evaporation of the liquid surface. Cells remain viable on the moist surface when the optimal collecting fluid is used, permitting prolonged sampling.

Portability

The sampler has been designed so that it has a smooth external surface with no projecting glassware which might be damaged by chance knocks. The rubber bungs and rubber-mounted suction tube act also as fenders, and more such protection could be added if desired. The thick-walled glass tubing has considerable mechanical strength, provided that the whole has been correctly annealed. The sampler will stand up to prolonged usage and repeated sterilization when reasonable care is employed and use is made of properly fitted storage boxes for transportation. It will stand firmly on a bench of its own accord but will not resist being dropped on a hard surface.

Nonspill Property

When the sampler is charged with sampling fluid and has the bungs in place, liquid cannot be spilled or transferred from one stage to another, however much the sampler is turned or inverted. The principle is similar to that of the unspillable ink-well.

Variations of the Basic Design

The basic design may easily be varied. In addition to the standard 55 liter per min model

(A in Fig. 2), two other models have been constructed (B and C in Fig. 2).

The 20 liter per min model (B) has stage characteristics somewhat different from A. Its first stage was designed to collect "fall-out" particles and has a 50% cut-off at 10 μ; the second stage has a similar cut off to the standard "pre-impinger" (50% at 4 μ), as described by May and Druett (5), so that its collection resembles upper respiratory retention, whereas the third stage resembles lower respiratory retention. The first-stage tube bore is 15 mm, and the jet impulse is so slight that no sintered disc is needed as the liquid surface remains undisturbed by the flow; nor is any filling hole required in the top stage as filling and emptying can be performed through the entry tube. A small dent in the glass floor of the top stage immediately under the entry tube acts as a sump. The clearance between the liquid surface and the bottom of the entry tube is 4 mm. The change in this as the liquid evaporates is unimportant. The second-stage jet diameter is 7.5 mm and the third, 2 mm. Each stage holds 4 ml of sampling fluid.

The small model (C), which is only 3.5 inches (8.9 cm) high, is operated at 10 liters per min, and was designed to have the same particle size range per stage as the large model (A). The jet bores are: first stage, 8 mm; second stage, 5 mm; third stage, 1.42 mm. The ratio of disc diameter to jet bore and the ratio of the clearance between the disc surface and the end of the tubes is as already described. This model is very much more compact than the standard Porton impinger which functions at about the same flow rate, yet it is capable of yielding more information and has the other advantages of the new design. It holds 2 ml of sampling fluid per stage.

Calibration and Testing

Particle Intake Efficiency

Wind-tunnel tests on the sampler were carried out by J. Edwards (*personal communication*). With the hemicylindrical baffle in place (17 in Fig. 1), the sampler was exposed to wind-borne aerosols of dyed, involatile, and uniform droplets generated by a spinning-top atomizer (8). An array of knife-edged isokinetic orifices around the sampler measured the absolute dosages. Estimations were by colorimetry.

Table 1 shows that the intake efficiency with the baffle in place is high, except for the largest particles and highest wind speed. The latter figures can be greatly improved by employing a larger baffle. For example, with no baffle, the intake efficiency was found to be only 9.6% at 15 μ and 10 mph but was raised to 99% by using

TABLE 1. *Per cent intake efficiency*[a]

Drop size	Wind speed				
	2 mph	5 m,h	10 mph	15 mph	20 mph
μ					
10	93 (0.3)	106 (0.7)	87 (1.5)	73 (2.2)	64 (2.9)
15	102 (0.6)	102 (1.6)	69 (3)	61 (4.5)	57 (6)
20	98 (1.1)	89 (2.8)	56 (5.6)	39 (8.3)	2? (11)

[a] Particle stopping distance (in millimeters) is given in parentheses.
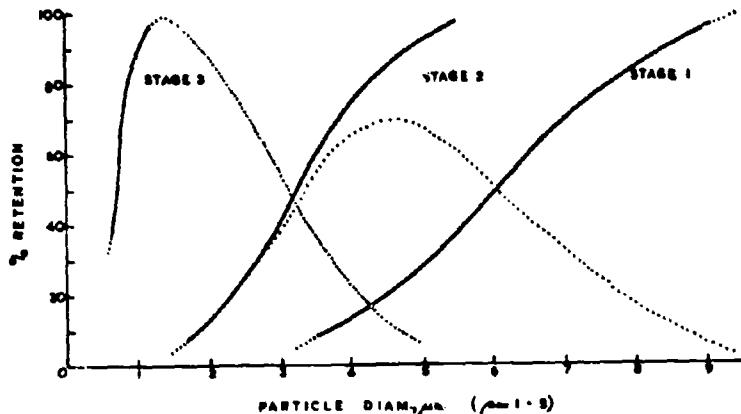
FIG. 3. *Individual stage cut-off curves (solid lines) and relative distribution of particles among all stages (dotted lines).*

a 6-inch square concave baffle, compared with the efficiency of 69% in Table 1, obtained with the Fig. 1 baffle. It is clear that a baffle is an essential addition to the sampler when there is a wind or cross draft. Note also that in Table 1 there is a good inverse correlation between the stopping distance (given in parentheses) and the intake efficiency, as predicted. With the particular conditions of the Table 1 tests, the intake efficiency is less than 50% when the stopping distance is greater than the radius of the intake tube.

Stage Cut-off Curves

It is essential to know the performance of each stage in terms of particles of each size retained; one must also know the relationship of this parameter to the dimensions of the jets and the throughput of sampled air. For this work, dry spherical airborne particles, nearly uniform in particle size, were generated from solutions of the intense blue dye Chlorazol Sky Blue (Imperial Chemical Industries Ltd.) sprayed from a spinning-top atomizer (8). The atomizer was mounted in a vertical wind tunnel similar to that described by Druett and May (3). In this, the up-

flow of air permits the rather large droplets generated to dry down to their final size without serious wall loss. The size of the dry particles is determined by the initial wet droplet size and the concentration of the dye solution. In this way, the range 2.5 to 10 μ was studied in small intervals, the relative proportion of particles caught in each stage being obtained by colorimetric estimation of the dye collected in the water with which the stage was filled. The spherical dye particles had about the same density as the particles obtained by spraying suspensions of bacterial cells (ca. 1.5 g/cc). The results for the standard 55 liters per min model are shown in Fig. 3, where the continuous lines are the cut-off curves for each stage considered individually, and the dotted lines (which in parts coincide with the continuous lines) show the percentage of all particles entering the sampler collected by each stage.

The cut-off curves of Fig. 3 are not as steep as those presented for the same optimal geometry by Mitchell and Pilcher (11) and by Mercer (9). There are two possible reasons for this. First, their methods were different, giving aerosols
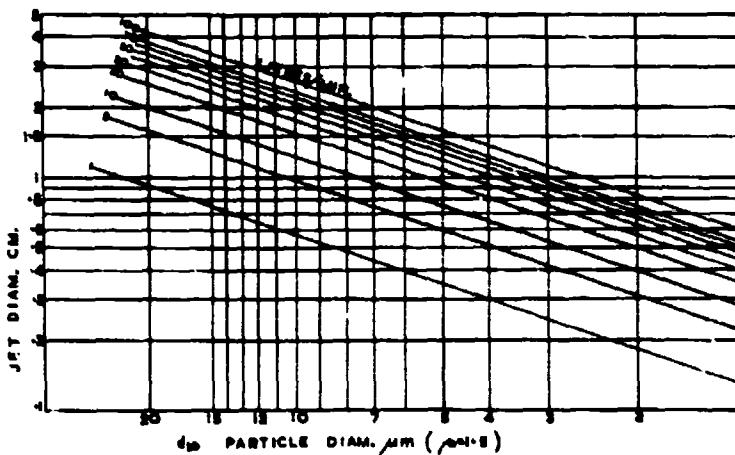
FIG. 4. *Relationship between jet diameter, particle diameter at 50% retention, and flow rate.*

which may have been more truly homogeneous in particle size than those used in the present work, where a spread in particle size tends to flatten the cut-off curves and reduces the precision. Second, the flow in the two upper chambers is rather complex. The air, after flowing out over the sintered discs, passes over a rather extensive liquid surface, and then turns upwards and inwards to enter the next jet tube. This causes turbulence and vortices which could throw out particles, though wall losses have not actually been noted other than to a limited extent in the tubes. Although the cut-off curves are less steep than hoped for, they are in fact as sharp as those of the preimpinger (5, 7) and the cascade impactor, and certainly not less sharp than the parts of the respiratory system that the sampler could simulate.

Effect of Varying Jet Sizes and Flow Rate

Workers wishing to adapt performance to their own requirements, with jet sizes or flow rates different from those described here, may use Fig. 4 as a guide. The chart derives from the expression for the dimensionless impaction parameters $P = k\rho V d^2/\eta l$, where k is a constant which in practice varies from system to system, ρ is the particle density, V the jet velocity, d the particle diameter, η the gas viscosity, and l the jet diameter (or characteristic length). For a given efficiency of impaction of particles, P is constant, and in the present case we are concerned with its value at d_{50}, the 50% cut-off diameter. For operation in air at normal temperature and pressure, we can also take ρ and η as constants so that, as in Fig. 4, we can relate d_{50}, l, and V (transformed into the more convenient flow rate parameter). The foundation of Fig. 4 was the set

of six values for d_{50} $(v/l)^{1/2}$ obtained from the calibration, as described in the preceding section, of the top two stages of the three models shown in Fig. 2. The values were 113, 108, 105, 122, 118, and 122 $\times$ 10^{-4} calculated in units of centimeters and seconds.

For any given value of d_{50}, the shape of the cut-off curve will be geometrically similar to those of Fig. 3.

Testing with Bacterial Aerosols

To study the performance of the sampler under rigorous conditions, a procedure identical to that previously described (6, 7) was adopted. In the relatively warm and dry conditions of summer daylight, a mixed suspension in known ratio of vegetative organisms (*E. coli*), which lose viability rapidly under such conditions, particularly in full sunlight, and of nondecaying spores (*B. subtilis var. niger*) was sprayed by a pneumatic hand spray and sampled at sufficient distance downwind for appreciable cell death to have taken place. By comparing the ratio of the two components recovered from the several stages of the sampler with their ratio before spraying, the percentage of *E. coli* cells remaining viable could be determined.

In the sampling array, two pairs of samplers were employed, each shielded from direct light Each pair consisted of a three-stage glass sampler and a "tilting" preimpinger plus impinger unit (7). The latter unit was known to have a high efficiency of intake of aerosol particles and was used as a standard reference sampler for comparison with the three-stage sampler. The two pairs were within a few yards of each other, close enough to ensure that they were exposed to comparable, but not necessarily the same, dosages.

TABLE 2. Dosages given by adjacent "tilting" and three-stage samplers in the field[a]

(1) Test no.	(2) Length of time airborne	"Tilting" sampler, total dosage (3) Escherichia coli	(4) Trace spores	Three-stage sampler[b] Dosage of E. coli cells (5) Stage 1	(6) Stage 2	(7) Stage 3	(8) Total	Dosage of tracer spores[c] (9) Stage 1	(10) Stage 2	(11) Stage 3	(12) Total	Percentage of E. coli remaining viable[d] (13) Stage 1	(14) Stage 2	(15) Stage 3
1	60 sec	22 / 24	76 / 128	14.3 / 20.5	2.1 / 9.5	3.1 / 5.0	19.5 / 35	46 / 52	16 / 40	24 / 17	86 / 109	31 / 41	13 / 24	13 / 29
2	70 sec	374 / 398	1,700 / 1,745	399 / 273	150 / 119	62 / 22	611 / 414	910 / 748	610 / 615	565 / 404	2,085 / 1,767	44 / 47	25 / 19	11 / 5.3
3	65 sec	259 / 249	1,559 / 2,160	390 / 220	121 / 118	93 / 64	604 / 402	933 / 625	298 / 364	398 / 255	1,629 / 1,264	42 / 35	40 / 31	23 / 25
4	55 sec	3,774 / 3,772	9,380 / 9,720	2,310 / 1,045	990 / 940	1,230 / 540	4,530 / 2,525	4,200 / 3,220	2,600 / 2,630	3,520 / 1,710	10,320 / 7,550	55 / 35	38 / 36	35 / 31
5	3 min	754 / 350	4,070 / 3,360	410 / 255	290 / 225	107 / 26	807 / 506	1,330 / 800	1,030 / 1,060	1,040 / 665	3,420 / 2,525	30 / 32	28 / 21	10 / 4
6	50 sec	2,550 / 2,588	8,940 / 8,630	1,315 / --[e]	510 / --	523 / --	2,348 / --	3,140 / --	1,820 / --	3,150 / --	8,110 / --	42 / --	28 / --	17 / --
7	4.5 min	19 / 28	198 / 208	25.8 / 35.5	16.7 / 11.5	6.1 / 2.2	48.6 / 49.1	77 / 122	64 / 60	61 / 33	202 / 215	34 / 29	26 / 19	10 / 7
8	60 sec	1,776 / 1,589	6,790 / 6,520	856 / 617	463 / 453	480 / 437	1,799 / 1,507	2,390 / 2,080	1,830 / 1,910	2,470 / 2,380	6,680 / 6,370	36 / 30	25 / 24	20 / 18
9	3.5 min	202 / 130	1,192 / 873	100 / 84	58 / 26	6 / 3	164 / 113	365 / 375	280 / 237	205 / 137	850 / 749	27 / 23	21 / 11	2.9 / 2.2

[a] "Dosage" in units of cells. Minutes per liter is the total number of cells in the sample divided by the sampling rate.

[b] The three-stage sampler recorded in the second line of each test was allowed to run for 30 min after receiving its sample. The other three samplers in each test ran for only about 5 min.

[c] The recorded tracer spore dosages have been adjusted to be equivalent to a starting ratio of unity for concentration of viable E. coli/tracer in the sprayed suspension.

[d] Column 13 is the percentage of column 5/column 9, and so on.

[e] Pump failed.

TABLE 3. *Atmospheric conditions during field tests of Table 2*

Test no.	Temp	Humidity (% RH)	Illumination perpendicular to sun (ft-c)	
	C			
1	17.8	56	8,000	(clear sky)
2	18.9	48	2,000–8,000	(5/10 cloud)
3	19.4	60–65	800–2,000	(overcast)
4	16.1	68	1,500	(overcast)
5	18.9	57–61	2,000–8,000	(5/10 cloud)
6	17.2	53	2,000–7,500	(5/10 cloud)
7	15.6	58	2,000	(overcast)
8	18.3	54	8,000	(clear sky)
9	19.4	54	8,000	(clear sky)

The first pair of samplers was switched off after sampling the aerosol for a few minutes, and in the second pair the three-stage sampler was left running for 0.5 hr to test whether the continuous aspiration of the dry air over the collected particles affected their viability. During this time, about 40% of the sampling fluid (phosphate buffer plus 1 M sucrose plus antifoam) evaporated.

Results

The results from nine tests are summarized in Table 2. Each test has two lines of dosage figures, one line for each pair of samplers. Table 3 gives the atmospheric conditions of the tests.

Effect of running for 0.5 hr. This may be judged by comparing the ratios of total dosages measured by each type of sampler in the first pair (top line of each test) with corresponding ratios in the second pair (bottom line) after extended operation of the three-stage sampler. If the extended operation caused a loss of viability, the ratio in the second pair would be less, on the average, than in the first. These ratios have been extracted, and are presented in Table 4 for the *E. coli* and in Table 5 for the spore tracer. Although in both tables the means from the second pair are slightly lower numerically, statistically they do not differ significantly from each other in either pair. Neither organism, therefore, shows appreciable loss after 30 min of aspiration in the conditions of these tests.

Overall recovery of viable cells. The intake and yield of viable cells in the three-stage sampler as compared with the tilting unit may also be judged from Tables 4 and 5. Means C and D in Table 5 are not significantly different from unity for the very robust spore tracer. We may conclude from this that both types of sampler in the pairs have the same intake efficiency and subsequently yield the same dosage of viable cells. In Table 4, the means A and B are significantly greater than

unity, indicating that the three-stage sampler yields more viable cells of *E. coli*. This is due to the absence in the three-stage sampler of the violent sonic velocity impingement in the Porton impinger, which is known to cause death of sensitive cells (6, 14). The tests do not tell us anything about the absolute recovery of *E. coli*, as one cannot assert from the figures presented that cells are not being killed in both samplers. However, in recent work by K. P. Norris (*personal communication*), the three-stage sampler in some winter conditions of less light, higher humidity, and long aspiration times has yielded viability figures for *E. coli* in the region of 100% on all stages. This indicates that there was no killing effect in the sampler. It is reasonable to conclude from accumulated experience with various other types of sampler, as well as with the new sampler, that neither the systems of impaction nor the washing-off process from the sintered discs results in appreciable loss of viable cells. Nevertheless, workers proposing to use the sampler with very delicate organisms would be well advised to carry out viability tests under their experimental conditions.

Effect of particle size on viability. As one of the main functions of this sampler is to yield estimates of organism viability within the various particle size classes, it is of value to examine the information in columns 13, 14, and 15 of Table 2, relative to this point. The periods during which the cells were airborne in the daylight was either close to 1 min or 3 to 4.5 min (column 2). The percentage of *E. coli* cells remaining viable after these periods has been averaged in Table 6. After 1 min, the small particles contained only half the proportion of viable cells that the large ones did. In the longer period, the small particles have continued to die off more rapidly, to about one-fifth the viability, on the average, of the large particle figure. In the full sunlight of the final test recorded in Table 2, nearly all the *E. coli* cells in small particles succumbed in 3.5 min.

DISCUSSION AND CONCLUSIONS

Morrow (12) has reviewed the recent position on the relationship of particle size in toxic dusts to respiratory deposition. He stresses the importance of selective sampling, which is usually designed to accept the small, lung-attacking particles and to reject the harmless large ones. For infective aerosols, selective sampling seems even more important because of two factors. First, it has been shown in the laboratory that with many infective diseases the smallest particles are the most dangerous, but it is also considered that in those infections which start in the upper respiratory tract,

TABLE 4. *Ratios of total Escherichia coli dosage in three-stage sampler to total in tilting sampler*

Pair	Test no.									Geometric means[a]
	1	2	3	4	5	6	7	8	9	
1	0.89	1.64	2.33	1.20	1.07	0.92	2.57	1.01	0.81	(A) 1.27
2	1.48	1.04	1.61	0.67	1.45	—	1.75	0.95	0.87	(B) 1.17

[a] Means A and B do not differ significantly from each other, but each is significantly greater than unity.

TABLE 5. *Ratios of total tracer spore dosage in three-stage sampler to total in tilting sampler*

Pair	Test no.									Geometric means[a]
	1	2	3	4	5	6	7	8	9	
1	1.17	1.22	1.02	1.10	0.83	0.91	1.02	0.97	0.72	(C) 0.98
2	0.86	1.01	0.59	0.78	0.75	—	1.03	0.98	0.85	(D) 0.86

[a] Means D and D are not significantly different from unity.

or in open wounds, etc., large particles are the most dangerous. Second, as Table 6 indicates, maintenance of viability in the airborne state may be markedly dependent upon the size of the airborne particle.

We therefore need to know the airborne concentration and state of viability of cells in various particle size ranges. It is not necessary that these ranges be narrow, sharply defined, and many in number, because physiological response to particle size effects cannot have sharp boundaries. For example, all sizes of particles can probably infect a wound, though large ones are much more likely to fall out than small ones; particles of say 6 to 7 μ are found in the alveoli, but those of 1 to 3 μ can penetrate in proportionately much greater numbers; persons differ in structure and breathing patterns, and flow rates vary widely. A selective sampler can, therefore, do no better than give a performance resembling a human average which can be generally accepted. The present model attempts this in its selection of three ranges: "large" particles, i.e., those greater than 6 μ in diameter (ρ = 1.5) which are normally retained in the upper respiratory tract; an intermediate range of 3- to 6-μ particles which are likely to lodge in the bronchi or bronchioles; and the fine range, 3 μ to single cells, which penetrate to the alveoli. Probably many bacterial particles are hygroscopic and will have time to increase in size in the depths of the lung. Lung retention of 1-μ viable particles should therefore be much higher than it is for dusts, many of which are expired at this size, so that the retention of 80 to 90% of single cells (*E. coli*) given in Fig. 3 seems reasonable. Single virus particles, ca. 0.3 μ and

TABLE 6. *Means of results from Table 2, columns 14, 15, and 16*

Stage	Percentage of *Escherichia coli* viable	
	at 1 min airborne	at 3 min plus
Top	38	29
Middle	27	21
Bottom	19	6

smaller, would not be retained in the sampler, whereas in the lung a high proportion would be retained by another process, diffusion to the walls. It seems unlikely, however, that such small isolated virus units can ever be generated in quantity by natural processes in air. The writer knows of no facts to confirm or refute this important point, which may be extremely difficult to resolve.

It is not claimed that the size ranges of the new sampler are necessarily the best. Further knowledge may require them to be altered in cut-off or in number, or both, and this can readily be done by use of the data in Fig. 4. The standard design might be modified by adding a top stage with similar characteristics to the top stage of model B, Fig. 2. This could distinguish large wound-infecting particles from respirable ones. In other work, a simple two-stage "upper" and "lower" respiratory tract simulation, as in stages 2 and 3 of model B, might be quite adequate. Selective sampling is based on the mass of work done on the effects of toxic industrial dusts on the relatively few workers exposed to them, whereas much less seems to be known about the equivalent

properties of the causative agents of airborne infection, to which all members of society are exposed. It is hoped that the new sampler, modified if necessary and used perhaps in conjunction with those of class I, will extend the latter field of knowledge.

The usefulness of the sampler is not necessarily confined to viable aerosols. It might well be used in air pollution work, indoors and out, and anywhere where respiratory irritation is related to particle size.

Finally, it may be mentioned that the gap between class I and class II samplers may be bridged by using soluble gel collection surfaces (gelatin with glycerol), which may afterwards be washed off in warm water for dilution and plating to give a single cell count. Both the Andersen (1) and Battelle (11) impactor configurations can easily be converted to this use. Studies on these lines were carried out in the present work, and were quite promising, except that the problem of finding a sufficiently stiff yet soluble gel to resist the high velocity impingement in the final stage was not solved. Microscope studies showed that impinged cells could not be effectively washed off agar surfaces, and dry surfaces cannot be used because of death of cells by desiccation. Also, the preparation of gel surfaces requires extra work, and it was concluded that the glass-liquid model is preferable because it offers greater simplicity in use, gives a reliable performance, and is inexpensive to manufacture.

AVAILABILITY

The sampler may be obtained from A. W. Dixon and Co., England. The design is patented (No. 1964 65) by the British Government.

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Air Sampling for Respiratory Disease Agents in Army Recruits

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INTRODUCTION

Among the few reports relating to the quantitative parameters of the transmission of many types of respiratory infection, are those of Riley for tuberculosis (4) and Tigertt et al. for Q fever (5). These reports suggest that adequate quantitative information can be obtained in natural situations only if it is possible to sample volumes of air which are very large in relation to the respiratory volume of man.

In a recent epidemiological study, we had the opportunity to employ the Large Volume Air Sampler (LVS) described informally by William Perkins at this meeting. The device has been described in detail by the manufacturer (Rept. 2586 of Litton Industries, Inc., Minneapolis, Minn.). The present report indicates a technological potential of importance, in spite of the preliminary nature of the data and the present lack of estimates of quantitative reliability.

EPIDEMIOLOGICAL PROBLEM

Acute respiratory disease (ARD) in Army recruits occurs in epidemic form in basic training centers all over the United States. The disease is caused mainly by adenoviruses, especially type 4, and occurs regularly each winter in new recruits during the 2nd, 3rd, and 4th weeks of the basic training cycle. Meningococci and group A streptococci are other common respiratory pathogens which may produce epidemic disease in recruits. Rates of infection and febrile illness vary during the year and from year to year, owing to a number of poorly understood factors, including physical and emotional stresses.

The pattern of a typical ARD outbreak at a training camp is depicted in Fig. 1. Adenovirus infections began to occur during the second week of training. Viral isolation attempts were performed at weekly intervals and yielded positive results from 37 of the 48 men (77%). Of the remainder, five had type-specific serum antibody at the time of first sampling and did not develop the infection, and six men showed rises in antibody titers, but without illness. Thus, of the susceptible subjects, all responded either by shedding virus or with antibody production. Twenty-seven of the infections were associated with febrile illness.

Bacterial studies of the throat and nasopharynx showed that meningococcal carrier rates among recruits increased from 42% upon entry in the Army to a peak of 90% during the 5th week of training. The curve for meningococcal incidence lagged about 1 week behind that of adenovirus type 4. We questioned whether adenovirus infections were responsible for the spread of meningococci in a manner analogous to the "cloud baby" spread of staphylococci (2). Sulfonamide-resistant strains of meningococci were initially absent, but accounted for 25 to 30% of strains isolated in the 6th through 8th weeks. Group A streptococcus carrier rates were rather stable and at a low level during the period of observation. (The excess cases shown in Fig. 1 in the final 2 weeks were associated with nontypable strains.) In the population under study, then, there were at least three different respiratory pathogens, each of which appeared to have a different pattern of transmission.

These recurring epidemiological features presented an excellent opportunity for the study of microbial transmission. The initial studies were concerned with detection of aerosols of the agents, which might serve as a source of infection. The LVS was employed in order to have maximal sensitivity of detection.

LVS STUDIES

In January 1966, during a period when attack rates were high, attempts were made to sample air in the vicinity of ill recruits. A number of patients were studied individually in a hospital room of 1,440 ft³ capacity. Attempts were made to isolate adenovirus type 4 and meningococci from throat gargles and throat swabs, respectively, and from the collecting fluid of the LVS.
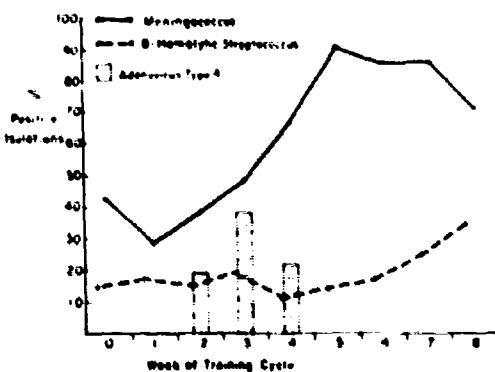
Fig. 1. *Pattern of a typical ARD outbreak among army recruits.*

In one such study, the patient was in the room for 10 min coughing frequently, before sampling began. A nasopharyngeal swab sample was found to be positive for group B meningococci, and a throat wash was positive for adenovirus type 4.

In 5 min of sampling, a total of 1,785 ft³ of air was drawn through the LVS, and the particulate content was collected in a total of 180 ml of sampling fluid. One group B meningococcal cell was recovered for every 99 ft³ of air, and 1 adenovirus unit per 277 ft³ of air. The latter unit was a tissue-culture infective dose employing 1 ml of sampling fluid as inoculum

Several attempts at sampling for adenovirus type 4 in barracks were made in training companies in which epidemic disease was beginning. Samples were collected during early evening when activity was at a peak, and again later after "lights out" when recruits were in bed. Results to date are incomplete. Data on adenovirus type 4 recoveries in two trials, however, are given in Table 1.

DISCUSSION AND CONCLUSIONS

These examples indicate that the LVS can provide bacterial and viral isolations from air collected in field situations. Meningococci were found in a concentration of one viable particle per 100 ft³ of air, whereas with adenoviruses one tissue culture infective dose was found in 300 to 3,000 ft³ of air. Although the results presented above can only be considered as preliminary data, they do indicate the need for sampling large volumes of air in studies of naturally produced aerosols. It is readily apparent that an all glass impinger, operating at 12.5 liters per min (6), is inadequate for collecting such low concentrations. These results may explain our failure in the past to detect infective particles in epidemiological sampling with an all glass impinger (1).

TABLE 1. *Results of air sampling for adenovirus type 4 in barracks*[a]

Trial no	Activity	Vol of air sampled ft³	Recovery
31	15 men making beds, coughing frequently	6,700	1 tissue culture infective unit[b] per 920 ft³
32	15 men in bed, coughing frequently	5,700	1 tissue culture infective unit per 2,830 ft³

[a] Room size, 13,347 ft³.
[b] Infected tissue culture after inoculation of 1 ml of sampler collecting fluid.

The experiments provide some of the information that Morton (3) had in mind in 1963 when he proposed four "postulates" relating to the epidemiology of airborne infection. They were as follows: (i) one must demonstrate the presence of airborne viable infective organisms; (ii) one must measure concentrations and particle sizes; (iii) one must demonstrate experimentally that concentrations and particles of this sort can cause infection; and (iv) one ought to show directly where the particles have come from. The present experiments show that the LVS can recover airborne, viable organisms at very low concentrations in natural aerosols. These studies have not demonstrated infectivity for man of the organisms collected, nor have they proved the source of the organisms.

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Discussion

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INTRODUCTION

In any discussion of air sampling methods, the practical consequences of the divergent interests of the participants quickly become apparent.

One group of workers, exemplified by the Microbiological Research Establishment at Porton, England, and by the Army and Navy Biological Laboratories here in the United States, are primarily concerned in the sampling of artificially generated clouds. This often involves higher air concentration, single species of microorganism, small particle sizes, and relatively little extraneous organic material.

Another, more diffuse group, to which I belong, is concerned with the sampling of the airborne flora of occupied places and the relation of this to infection and disease in ordinary life.

The airborne organisms of interest in these environments are usually present in small numbers only, accompanied by much larger numbers of a wide variety of other species; particle sizes extend above 20 μ equivalent particle diameter, and the typical particle is probably principally composed of dried secretion or cellular debris.

In spite of these differences, however, the technical problems involved have sufficient fundamental similarity for me to hope that the topics I am going to discuss briefly are of concern to both.

COLLECTION OF THE SAMPLE

Separation of the Particulates from the Air

This is the primary problem of all air sampling. The physical principles involved are now generally understood (3), but there are two particular points worthy of consideration.

First, when looking for a particular species in high dilution, it is helpful to collect the organisms into as small an amount of medium as possible. The level of interest may be as low as one or two viable cells in 100 ft³ of air or more. We have experimented on two approaches with a view to sampling for respiratory virus. In contrast to May's experience (10), we have found it possible to recover the great majority of the cells collected by impingement onto an agar surface by washing with a small volume of fluid while rubbing with a glass rod, both with and without previous coating of the agar surface (6). Alternatively, the airborne particles have been impinged onto the surface of a stainless-steel cylinder which is kept moist by continual rotation in fluid medium (Fig. 1). A more complex principle, which has been em-

ployed in some commercial air cleaners (Cyclone type air cleaners with separated air-flow—Rotonamic, Farr Co., Los Angeles, Calif.), is to concentrate the particulate material into a part of the air stream which is then bled off into the sampler proper.

The second general point concerns the provision of efficient, quiet air suction devices. The energy required to extract 10 liters of air per min against 10 cm of water pressure is only ⅙ of a watt. Even 600 liters per min at 30 cm of water pressure represents only 30 w. (These figures represent the two extremes in the air samplers we use.) Measured against these figures, the pumps we commonly employ are both noisy and very inefficient. At the lower end of the scale, we have just succeeded in building a pump with a 4-inch neoprene diaphragm having a mechanical efficiency of about 60%.

Its present motor, about 35% efficient, takes 150 ma at 6 v or 0.9 w, an overall efficiency of nearly 20%. Motors with 60 to 70% efficiencies are now available in this size. A quiet, efficient pump handling several hundreds of liters of air per minute at back pressures up to 25 to 35 cm of water would be a real asset.

SIZE DISTRIBUTION OF THE PARTICLES

Selective impaction, either onto solid or liquid medium, is probably the best method we have. Very elegant methods employing sedimentation over fixed distances through nonturbulent air streams, sometimes in a centrifugal field, have been employed for dust analysis (14, 15), but not, so far as I know, for microorganisms. These lead to much sharper size separations than the impaction devices, where the overlap between the size groups is uncomfortably large. The restricted working size range is a further limitation to several designs. In occupied places, microorganisms are commonly associated with particles whose median equivalent diameter is about 12 to 15 μ (11). Such clouds can only be adequately sized if the first stage cut-off of the sampler approaches 20 μ or larger.

COMPOSITION OF THE PARTICLES

This is not often considered as a sampling problem, but it may often have important epidemiological implications. Rubbo and his co-workers (13) attempted to demonstrate an association between cotton fiber and airborne bacteria in hospital
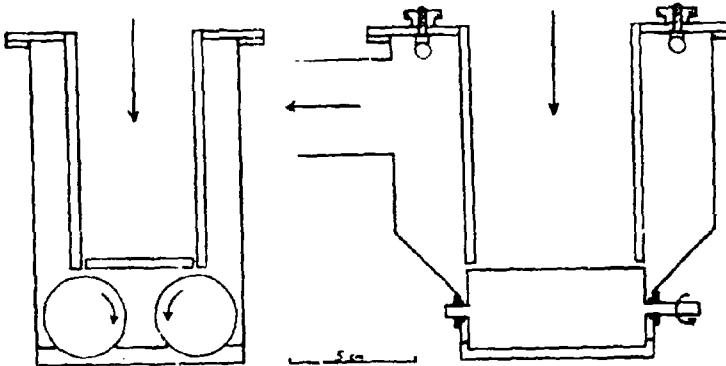
FIG. 1. "Stamp-licker" air sampler; two sections at right angles. The perspex ent. y duct terminates in two slits 1 mm wide by 5 cm long, each with their lower faces 2 mm above the top of a stainless-steel cylinder 2.5 cm in diameter. The matt-surfaced cylinders are rotated in polytetrafluoroethylene bushes by an external motor at 6 rev/min and dip into the collecting fluid. The main body of the sampler is fabricated from stainless-steel sheet, and the entry duct is clamped down onto locating blocks, with a sponge rubber sealing gasket. Directions of air flow and of rotation of the cylinders are shown by arrows.

wards. Davies and Noble (4) suggested that Staphylococcus aureus is often found associated with skin fragments. Some indication of the effective disseminating sources of, for example, Clostridium welchii might be found along these lines. Another aspect of the composition of the airborne particles is the number of viable cells contained in it. The clumps of cells in the larger particles of artificially generated clouds will often be dispersed in liquid medium. In this case, comparison between colony counts obtained by impaction on solid media and the recovery from fluids enables an estimate of the mean number of viable cells per clump to be obtained. Species, such as certain streptococci, whose cells do not separate easily during multiplication, obviously cannot be examined in this fashion. More generally, it appears that the viable cells in many, if not most, naturally occurring particles cannot be easily separated (1). Dispersion by mechanical methods or by chemical (e.g., enzymatic) action has been suggested but not adequately explored (7, 9). Estimation of clump size from the shape of the dose-survival curve resulting from exposure to a sterilizing process with a constant chance of cell death per unit dose increment is essentially laborious, and a suitable sterilizing process cannot always be found. Electron bombardment has been used for staphylococcus-carrying particles (8), but the killing action of this and other radiations is often complex. Knowledge of the clump size is important in infectivity studies. The dose may differ substantially from the particle estimate, and this difference may vary with, for example, the age of the suspension, as death of cells reduces the average number of viable units per particle.

SELECTIVE CULTURAL METHODS

The viable cells carried on dry airborne particles differ from those present in liquid cultures. They appear to be more resistant to the action of ultraviolet radiation, although it is not clear to what degree this is a property of the cells themselves or to what degree it depends on the nature of the matrix in which they are embedded (2). They may differ in infectivity and virulence (5, 12), and they may differ in antigenic structure and power to combine with immunofluorescent reagents. More pertinent here, they are often less able to grow on selective media (16). How far this relative disability is affected by a preliminary hold time in a collecting fluid I do not know. In practice, this cell defect, whatever its nature or cause may be, is a severe limitation. Sometimes a useful discrimination in favor of the species of interest without significant loss can be obtained by reduction in the concentrations of the selective agents. An alternative approach is to grow the organisms to microcolony size on an unselective medium and then to replicate onto one or a variety of selective media (16). The transferred cells are then in their growth phase and are better able to continue growth in the inhibitory medium.

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Assessment of Experimental and Natural Viral Aerosols

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INTRODUCTION

This report describes a number of characteristics of artificially prepared aerosols containing coxsackievirus A, type 21, a virus that causes respiratory illness in man. Studies on natural aerosols produced by subjects who have been infected with this virus are also described. The findings are part of a continuing program of investigation of the role of aerosols in human viral respiratory disease conducted as a joint undertaking by the U.S. Army Biological Center, Fort Detrick, Frederick, Md., and the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

The report is divided into two sections. The first deals with observations on the properties of laboratory-generated viral aerosols used for inoculation purposes, and the second covers the production of viral aerosols by experimentally infected subjects and the contamination of air in rooms occupied by them.

The program has availed itself of a large body of information concerning bacterial aerosols and was aided by some new techniques pertinent to viral aerosols. The work so far has provided a sound experimental basis for a broad approach to the problem of the role of viral aerosols in human respiratory disease, and the information already gained has indicated a possible significance for this mode of dissemination of these infections.

RESULTS

Preparation and Properties of a Small-Particle Viral Aerosol

Studies with artificially prepared small-particle aerosols were undertaken to provide better control of the site of inoculation than was possible with liquid suspensions instilled into the noes.

Opportunity was also provided to make observations on virological and physical properties of this form of viral suspension. The results to date are limited to findings with coxsackievirus A, type 21, but the methodology is applicable to agents belonging to three other major virus groups: adenoviruses, rhinoviruses, and influenza viruses.

An aerosol apparatus originally designed for use with a bacterial organism (5, 8, 11) and the Collison atomizer (2, 9) were selected for evaluation. The aerosol was generated from a safety-tested, tissue culture suspension of virus (4, 10). The equipment produced a heterogeneously sized, small-particle aerosol under the conditions in which it was used. The sampling instrument used in these studies was the Shipe impinger (16). It contained 5 to 10 ml of a suitable cell culture medium that could be used directly in the selected assay system. The high efficiency of the Shipe impinger for the collection of virus from these aerosols has been established. About 50% of the total virus atomized was recovered.

Preliminary experiments were performed to determine the relationship between the concentration of the viral suspension to be sprayed and the viral concentration of the resulting aerosol. This information was essential to provide a degree of control over doses of virus to be administered. Figure 1 shows data collected with coxsackievirus A-21. It is apparent that a direct relationship exists between the concentration of the virus in the spray suspensions and that of the aerosol. With this information, it was possible to estimate, within an acceptable range, doses of virus to be administered to volunteers by appropriate dilution of the spray suspension. The actual dose administered was determined at the time of each inoculation (4).

Another factor of concern with both the experi-

mental and natural aerosols was the distribution of virus in aerosols of heterogeneous particle size. It was important to know whether virus concentration followed the volume distribution of the aerosol or whether some unknown selective force caused an unexpected concentration of virus in particles of one size or another. To answer this question, the concentration of virus was measured in aerosol particles of various size ranges. The particle-size distribution of the aerosol was determined by direct microscopic measurement, and virus was collected in an Andersen sampler (1). The plates were prepared by pouring a 21-ml base layer of hard agar and, after this solidified, an overlay of 6 ml of 12% gelatin was added (6). The agar served to place the gelatin surface at the proper level below each sieve plate. After sampling, the gelatin in the plates was liquefied at 37 C and was removed for virus assay. Figure 2 shows the results of one of these experiments. As can be seen, the virus concentration appeared to be more closely related to the volume distribution rather than the particle number distribution of the aerosol. Similar findings (1) have been reported for bacterial aerosols.

Particle sizing of virus aerosols, both experimental and natural, presented no unique problems. Standard techniques with use of cascade impactors, membrane filters, and settling slides were used without modification (14).

Viral Aerosols Produced by Infected Persons

For present purposes, natural aerosols are defined as those arising directly or indirectly from infected volunteers. The events that were considered to be possible sources of viral aerosols
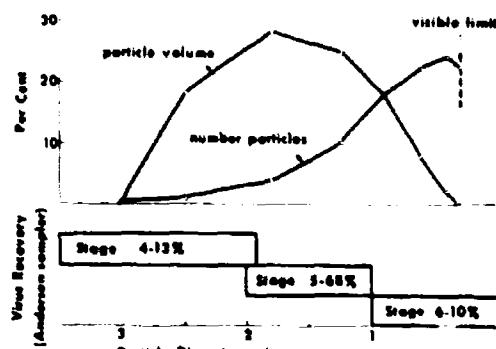
FIG. 2. *Distribution of coxsackievirus A-21 in an aerosol heterogeneous in particle size. Reproduced by permission from reference (4).*

included sneezes, coughs, talking, and breathing. Because talking and breathing produced relatively few particles, our studies were concentrated on the sneeze and cough.

Two procedures were devised to examine the aerosols produced in coughs and sneezes by infected volunteers. One was used to recover virus from coughs and sneezes, whereas the second was principally concerned with sizing and distribution of particles in the aerosol.

Recovery of virus from aerosols and droplets produced by coughs and sneezes was accomplished by having the volunteer sneeze or cough into a deflated weather balloon (Fig. 3). The balloons were washed several times to remove as much talc as possible. They were sterilized while submerged in buffered saline and then stored in a refrigerator. Prior to use, the excess fluid was removed and replaced with 10 ml of cell culture medium. The balloon was attached to a face mask that provided a tight fit around the nose and mouth of the volunteer. After the volunteer sneezed or coughed, the neck of the balloon was clamped off. By use of a Shipe impinger, the air phase of the balloon was immediately sampled. The inlet on the critical orifice was modified from the usual blunt-end capillary to a funnel shape to reduce the loss of larger particles ($>5\ \mu$) by impaction (12). The balloon was reinflated with laboratory air, and the wall inside was carefully rinsed with 10 ml of medium. The impinger fluid was assayed for virus directly. The wash medium from the balloon was clarified by centrifugation, and the supernatant fluid was assayed for virus. This procedure gave the approximate amount of total virus in a sneeze or cough, and roughly defined the airborne component as distinct from the portion that either impacted on the inner wall of the balloon or immediately fell out because of large-particle size.
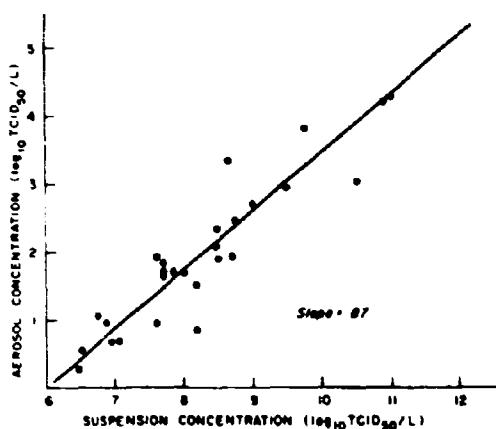
FIG. 1. *Relationship of coxsackievirus A-21 concentrations in spray suspensions and aerosols. Reproduced by permission from reference (4).*

FIG. 3. Use of a weather balloon for the entrapment of sneezes and coughs.

Some examples of results obtained by use of this technique on volunteers infected with coxsackievirus A-21 are given in Tables 1, 2, and 3. These results are presented to illustrate that the procedure can be used for detecting virus in these expiratory events. Although the quantities of virus recovered range from a few TCID$_{50}$ to several thousand, the results cannot be considered in absolute quantitative terms. There is little doubt, however, that virus can be aerosolized in the process of sneezing or coughing, and that, in some instances, sufficient quantities are expelled which could account for infection of susceptible individuals in the environment.

Particle-size analyses were made on sneezes and coughs collected in a 127-liter stainless-steel chamber. The chamber was shaped as a truncated cone to minimize impaction of particles on its sides (Fig. 4). It was equipped at the small end with a pneumatic tube that tightly fit the facial contour around the nose and mouth. At the opposite end of the chamber were several sampling ports that would accommodate impingers, impactors, Andersen samplers, and a particle-size analyzer (13). A large weather balloon could be inserted into the chamber with its mouth open to the outside. This balloon would inflate as the aerosol was sampled,

avoiding the dilution of the aerosol with outside air. Preliminary particle-size analyses showed that the particle content of room air obscured the particles produced by the sneeze or cough. To circumvent this problem, the volunteer was placed in a plastic tent that was continuously purged with filtered air, as was the chamber. After several minutes of deep breathing in this environment, the particles were almost completely removed and reliable measurements could be made.

An example of the particle-size distribution of aerosols from sneezes and coughs, by use of this equipment, is shown in Table 4. In comparing the sneeze and cough from a single volunteer, it may be noted that the particle-size distributions were similar. The sneeze produced 18 times more particles than did the cough. The volume of the sneeze was about 30 times that of the cough.

Particles above 15 μ in diameter presented a special problem which has not been successfully solved. Because of their high settling rate and low concentration, no attempt was made to enumerate these particles.

Air Sampling in the Environs of Infected Volunteers

After it was established that the infected human volunteer did produce airborne virus, it was of interest to determine whether virus could be recovered from the room air surrounding the subjects. Preliminary calculations were based on the average volume of oral secretions in a sneeze, the expected titer of virus in oral secretions, and the volume of the room. If volunteers harbored 10^4 TCID$_{50}$ of virus per milliliter of oral secretions, sneezed 100 times in a closed room (70,000 liters in volume), and atomized 5.9×10^{-4} ml of secretions with each sneeze, 12,000 liters of air would have to be sampled to recover 1 TCID$_{50}$ of virus. Any biological and physical losses of airborne particles would tend to increase the volume of air that must be sampled. It was apparent, therefore, that devices that sampled 10 to 30 liters of air per minute were impractical for use in these studies. This eliminated from consideration virtually every commonly used sampling device.

The apparatus that was selected for these studies was a newly developed large-volume sampler (LVS; designed by Litton Systems, Inc., Minneapolis, Minn., under contract with Fort Detrick) that functioned by electrostatic precipitation (Fig. 5 and 6). It is capable of drawing air flows up to 10,000 liters per minute. The air passes through a high-voltage corona that charges particulate matter, causing it to precipitate on a grounded disc. The disc rotates at 200 to 300 rev/min and is covered with a thin, flowing film of collecting fluid. The diluent used in

TABLE 1. *Recovery of coxsackievirus A-21 from coughs of volunteers by use of the balloon technique*

Volunteer no.	Source	TCID50 of virus							Positive tests[b]
		4 days[a]	5 days	6 days	7 days	11 days	14 days	29 days	
1	Air	30	48	25	0	25	10	0	6/7
	Wall	0	0	260	30	0	0	0	
2	Air	90	0	0	0	0	0	—[c]	1/7
	Wall	0	0	0	0	0	0	—	
3	Air	0	0	0	0	0	0	—	1/7
	Wall	0	0	0	30	0	0	0	
4	Air	90	0	0	10	0	0	0	2/7
	Wall	0	0	0	0	0	0	0	

[a] Days after exposure.
[b] Number of positive coughs/total tested.
[c] Not tested.

TABLE 2. *Shedding of coxsackievirus A-21 by human volunteers*

Volunteer no.[a]	Specimen	TCID50					Positive/total[c]
		3 days[b]	4 days	5 days	6 days	7 days	
1	Oral secretion[d]	>32,000[d]	30	100	3,200	100	5/5
	Cough						
	Air[e]	90	0	0	0	0	1/5
	Wall[e]	30	0	0	0	0	
	Sneeze						
	Air	—[f]	0	0	0	—	2/3
	Wall	—	0	30	15	—	
2[g]	Oral secretion			0	100	3,200	2/3
	Cough						
	Air			5	15	0	2/3
	Wall			0	0	0	
	Sneeze						
	Air			0	0	90	1/3
	Wall			0	0	800	

[a] In a third volunteer, all specimens were negative (not infected).
[b] Days after exposure.
[c] Number of positive specimens/total tested.
[d] TCID50 per 0.2 ml of secretion.
[e] Balloon technique (see text).
[f] Not tested.
[g] Began shedding virus on day 5.

our experiments was Eagle's basal medium containing 20% calf serum, and antibiotics to reduce bacterial and fungal contamination. About 125 ml of medium was recirculated through the apparatus. Evaporation over a 3.5 min period caused a loss of about 25% of the fluid.

Preliminary tests to determine the efficacy of the sampler were carried out in a room with a volume of 32,800 liters. A suspension of coxsackievirus A-21 was atomized into the room by a University of Chicago Toxicity Laboratories (UCTL) atomizer (15), and the aerosol was circulated by a 15-inch fan directed toward the aerosol stream at a 90° angle (Fig. 7).

Since most determinations were made on aerosol concentrations below the threshold of other sampling devices, there was no base line for comparison. It was necessary, therefore, to calcu-

TABLE 3. *Shedding of coxsackievirus A-21 by human volunteers*

Volunteer no.	Specimen	TCID50					Positive total[b]
		3 days[a]	4 days	5 days	6 days	7 days	
1	Oral secretion	10,000[c]	1,000	1	10	100	5/5
	Cough						
	Air[d]	10	150	0	0	0	2/5
	Wall[d]	0	400	0	0	0	
	Sneeze						
	Air	--[e]	0	0	0	0	0/4
	Wall	--[e]	0	0	0	0	
2[f]	Oral secretion		0	100	10	0	2/4
	Cough						
	Air		10	480	0	0	2/4
	Wall		0	80	0	0	
	Sneeze						
	Air		0	4,800[g]	0	9	2/4
	Wall		0	500,000[g]	0	1,600	
3[h]	Oral secretion				1	10	2/2
	Cough						
	Air				15	0	2/2
	Wall				160	30	
	Sneeze						
	Air				-	0	0/1
	Wall					0	

[a] Days after exposure.
[b] Number of positive specimens/total tested.
[c] TCID50 per 0.2 ml of secretion.
[d] Balloon technique (see text).
[e] Not tested.
[f] Began shedding virus on day 4.
[g] Gross nasal secretions were expelled by the sneeze.
[h] Began shedding virus on day 6.

late the efficiency of the apparatus from the amount of virus atomized. Figure 8 shows the results of these experiments. Recoveries ranged as low as 0.6% to as high as 71%, with the vast majority falling between 1 and 20%. It is significant that virus was recovered in all experiments in which the predicted aerosol concentration was 0.001 tissue culture infectious unit (TCIU) per liter or greater. [Concentration was estimated by the dilution method of Fisher and Yates (7).]

In trying to establish the best method for handling the fluid from the LVS prior to assay, a number of techniques were employed in an effort to concentrate the virus and reduce the problem of contamination. These included both high- and low-speed centrifugation, sonic disruption, extraction with trichlorotrifluoroethane, and sometimes no treatment at all. Although these procedures were more or less successful in reducing contamination or reducing the volume of fluid to be tested, they did not seem to alter the per cent recovery.

In the interpretation of these recovery values, several factors must be considered:

(i) The sampling period was based on one turnover of room air through the sampler. Since the effluent air was returned to the room, the maximal efficiency would not be expected to exceed 66%.

(ii) No measurement of biological or physical loss of the aerosol was made. Any losses of this nature would reduce the maximal per cent recovery that would be expected.

(iii) When contamination of the cell cultures occurred, the tubes were eliminated from the assay, and it was noted that a low recovery value was obtained in these instances.

A second series of experiments was done in a similar manner, except that a tracer, sodium fluorescein, was incorporated into the virus suspension to be atomized, and large concentrations of virus were used. With these large concentrations of virus, it was possible to make direct comparisons between the LVS and the Porton all-glass impinger (AGI), a common laboratory

FIG. 4. A stainless-steel, 127-liter chamber for the collection of sneezes and coughs.

TABLE 4. Airborne portion of a representative sneeze and a representative cough

Particle diam	Sneeze		Cough	
	No. of particles	Vol	No. of particles	Vol
<1-1	800,000	167,000	66,000	13,860
1-2	686,000	1,210,000	21,300	37,701
2-4	101,000	1,427,000	2,800	39,564
4-8	16,000	1,800,000	700	79,100
8-15	1,600	1,270,000	38	30,248
Total	1,604,600	5,874,000	90,838	200,473

* Ratio of number of particles in a sneeze to number of particles in a cough was 17.6:1; the ratio of volume of a sneeze to volume of a cough was 29.3:1.

aerosol sampler. The LVS was operated for a 3.5-min period, whereas the AGI were operated for 1 min (12.5 liters per minute of flow). Based on the total amount of virus and fluorescein aerosolized into the room and the amounts recovered
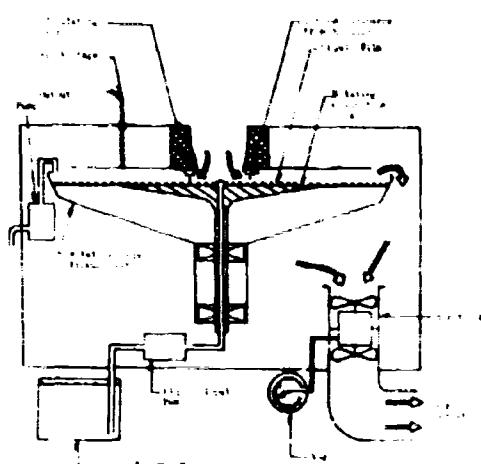
FIG. 5. Schematic diagram of the air and liquid flow systems of the large-volume air sampler.

in the samplers, recovery rates were calculated. Table 5 shows that the LVS consistently recovered more fluorescein than the AGI. The virus recovery
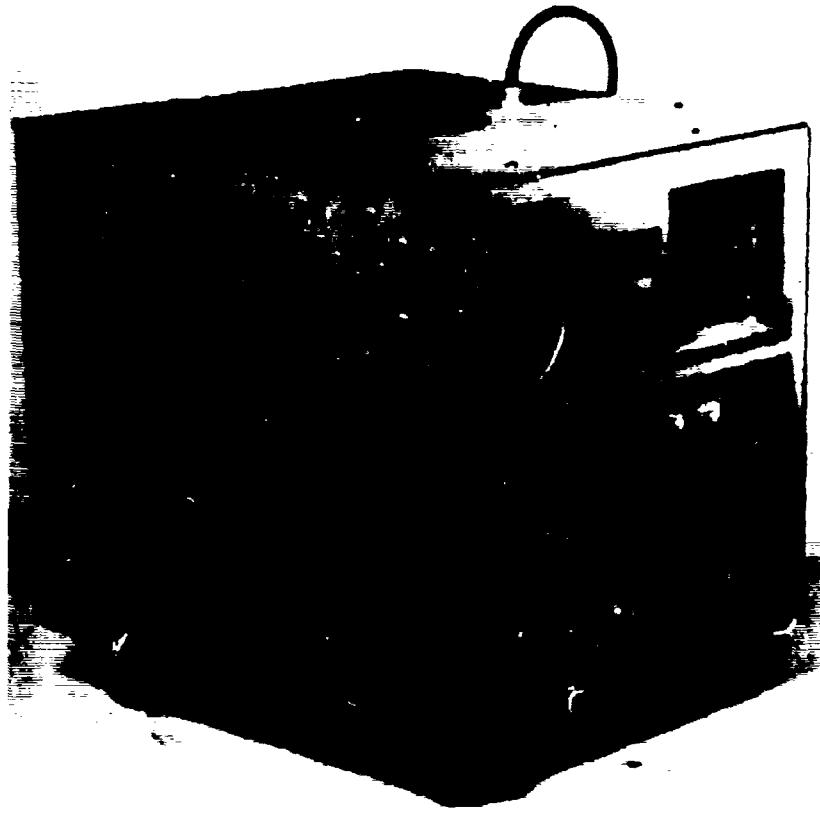
FIG. 6. *Photograph of a large-volume air sampler.*
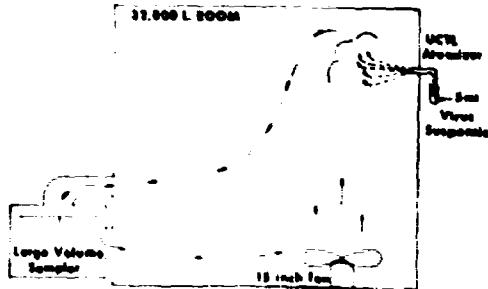
FIG. 7. *Sampling arrangement for testing the efficiency of the large-volume air sampler.*

rates exhibited variability between samples. It was also significant that the recovery rates of the samplers were not changed in situations where sampling was started after the aerosol generator was stopped. These results suggest that the LVS is a highly efficient sampler and that biological inactivation of the virus did contribute to the low recoveries in earlier experiments (Fig. 8).

The large-volume sampler was used for the detection of virus in the air of rooms occupied by volunteers experimentally infected with aerosols of coxsackievirus A-21. Prior to sampling, the
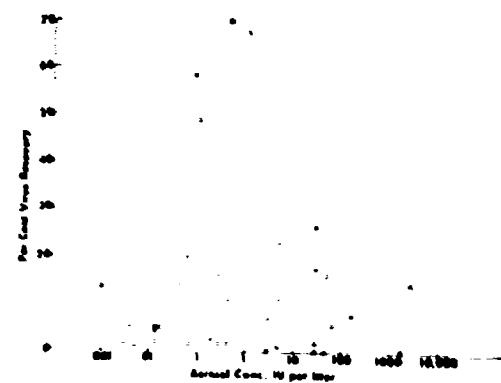
FIG. 8. *Recovery of coxsackievirus A-21 from aerosols of varying concentrations by use of the large-volume air sampler.*

ventilation was turned off for a 2- to 4-hr period. The room was closed except for entry for the sampling. During the 2- to 4-hr period, no restrictions were imposed on the volunteers, and routine activity was normal. The sampler was operated for a 12-min period, which amounted to sampling 120,000 liters of air. The room volume was 70,000 liters. It was estimated that about 82% of the room air was sampled by this procedure. The sampling fluid was immediately frozen and stored for subsequent assay in cell cultures.

The results of one experiment in which two rooms were sampled twice daily for 5 days are shown in Table 6. Virus was recovered from 5 of these 16 samples tested. Overall recovery rates revealed a distinct relationship between the quantity of virus in secretions and recovery of virus in the LVS (3).

DISCUSSION

The purpose of these studies was to describe procedures employed in studies on the role of viral aerosols in human viral respiratory disease. The results showed that viral aerosols prepared with the Collison atomizer can be adjusted to a desired content of virus, and that the size distribution of such aerosols coincides to most particles produced in sneezes and coughs from infected

TABLE 5. *Recovery of coxsackievirus A-21 and fluorescein from room aerosols*

Exp no	Conditions of sampling	Sampler	Per cent recovery	
			Virus	Fluorescein
7	During spraying	LVS	1.2	64
		AGI₁	2.5	46
		AGI₂	6.0	45
		AGI₃	2.5	41
8	After spraying	LVS	16.0	64
		AGI₁	0	42
		AGI₂	32.0	43
		AGI₃	16.5	39
11	After spraying	LVS	18.8	74
		AGI₁	2.5	42
		AGI₂	3.0	47
		AGI₃	2.5	47
12	During spraying	LVS	7.0	65
		AGI₁	5.4	46
		AGI₂	3.0	52
		AGI₃	2.5	50
Avg		LVS	10.75	66.8
		AGI	7.13	45.0

TABLE 6. *Recovery of coxsackievirus A-21 from room air by use of the large-volume sampler*

Room no.	Sampling time	Titer of virus by days after exposure				
		3	4	5	6	7
211	7:00 AM	*	0	185	5	0
	10:00 PM	*	0	0	0	–
	No. positive/ no. tested[b]	1/*	1/3	2/3	2/3	2/3
215	7:00 AM	*	0	0	90	90
	10:00 PM	0	*	75	0	
	No. positive/ no. tested[b]	1/3	2/*	3/3	3/3	3/3

[a] Not done.
[b] Number of volunteers having virus-positive saliva, cough, or sneeze, or all three, over total in the room.

volunteers. Thus, the convenience and precision of the technique and its resemblance, at least in part, to natural viral aerosols indicate its potential utility for studies of this kind.

Virus was recovered from coughs and sneezes by collection in a weather balloon. The disadvantages of this procedure were that only a rough approximation of airborne virus could be obtained and that it was not practical to measure the size of the airborne particles.

The particle-size studies were best performed in a rigid, stainless-steel chamber. These were accomplished by a combined use of a cascade impactor and a particle-size analyzer. The larger particles were not measured by these procedures, because they did not remain airborne long enough and because they were present in relatively low concentrations.

The use of a large-volume sampler to detect virus aerosols in room air proved to be useful, and the presence of virus in the environmental air of infected subjects was demonstrated. When these studies were performed, the apparatus was used essentially as it was originally designed. It is conceivable that, with additional work and modifications, the LVS can be used for quantitative determinations of airborne virus in a natural environment. In this regard, it was of interest to find that the greatest number of positive LVS samples occurred in the room with patients that shed the larger amount of virus (3). With due regard to the inefficiency of present recovery methods, evidence given here and from another study from this laboratory (4) suggests that infected persons may discharge sufficient virus into their environment to account for airborne transmission of this disease.

The full significance of these studies will not be realized until investigations of this nature are extended to other respiratory virus diseases. By examining viruses of varying epidemic potential and comparing such factors as infectious dose, clinical illness, virus-shedding patterns, airborne survival, etc. on a quantitative basis, a better knowledge of the underlying mechanisms of airborne transmission of virus will be gained. This information will be helpful in approaches to environmental control of respiratory disease.

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Discussion

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Gerone and his associates have presented observations on the production of small-particle virus aerosols with a Collison atomizer in a modified Henderson apparatus, information on the production of viral aerosols by persons infected with coxsackievirus A-21, and data acquired by large-volume air sampling in the environs of infected volunteers. Portions of those studies have been published in greater detail elsewhere (1, 2).

From these observations, certain inferences are made as to the significance of small particles in the transmission of naturally occurring disease due to coxsackievirus A-21, as to the suitability of

the exposure method for the inoculation of volunteers, and as to the usefulness of the large-volume air sampler in demonstrating virus in the environment of infected persons.

The purposes of large-volume air sampling may be twofold. First, one might theoretically detect airborne agents in one-thousandth the concentration detectable by conventional samplers. Second, the larger volume sampled might give one more confidence in an estimate of concentration than in that derived by the smaller sample size conventionally obtained. The large-volume sampler has two inherent limitations. First, because of problems of evaporation of collecting fluid, only relatively short periods of sampling are possible. Second, no estimate is possible of particle size distribution of the aerosol sampled. The first problem might be solved by the introduction of sterile distilled water at a rate equal to the loss by evaporation, thus maintaining the integrity of the composition of the collecting fluid. The second, that of particle sizing, appears to be insoluble with this equipment.

The results presented indicate that the device has not attained its theoretical capability to meaningfully quantitate airborne virus. A 100-fold variation between estimates of the concentration of a given aerosol and a 20-fold average variation over the concentrations sampled reduce the device in its present state to a qualitative sampler whose negative results would be suspect.

The presented results of comparison of the large-volume sampler with the Porton all-glass impinger raise more questions than they answer. Greater than 10-fold differences in virus recovery versus tracer recovery are indicated for both samplers, under conditions where tracer recovery was remarkably consistent. These are not compatible with the stated accuracy and reproducibility of the virus assay procedure (standard deviation, 0.25 $\log_{10}$ TCID$_{50}$ per ml).

The studies on sneezes and coughs establish two main points. First, the particle size distributions and particle volume distributions are markedly unlike that of the artificially generated aerosol used to infect volunteers. Second, no correlation can be made between titer of oral secretions and the amount of virus in a sneeze or cough.

The suitability of any method for the inoculation of volunteers by inhalation can be defined in terms of predictability of the dose to be administered and the site of deposition desired for the purpose of the experiment being conducted.

Predictability of the dose administered to man is influenced by stability of the agent in the spray suspension and in the airborne particulates gener-

ated, the uniformity of the aerosol generated, both qualitatively and quantitatively, and by the rate, manner, and volume of breathing of the test subject. It is also obviously dependent upon the accuracy of the assay procedures employed.

The lengthy training required to accustom volunteers to the highly stylized breathing cycle of nasal inhalation and oral exhalation required for mask exposures has long been recognized. Without this, marked variation in respiratory rates and tidal volumes will materially affect sites of deposition of airborne particles, yet not be reflected in the presented dose.

Data have been presented showing a not unreasonable relationship between the concentration of coxsackievirus A-21 in spray fluids and in the aerosol generated in the device employed. The inconstancy of the relationship, as demonstrated by these data, deserves consideration. As examples, aerosol concentrations of approximately 10 TCID$_{50}$ per liter were obtained with spray suspensions with concentrations ranging from 6.9×10^{10} to 8.2×10^{10} TCID$_{50}$ per liter, and aerosols containing approximately 1,000 TCID$_{50}$ per liter were obtained from suspensions containing 8.6, 9.5, and 10.5×10^{10} TCID$_{50}$ per liter. Conversely, from a single spray fluid concentration were generated aerosols containing less than 100 and over 1,000 TCID$_{50}$ per liter. Thus, although the relationship, or so-called "spray factor" may be useful in generalized predictions, it does not have the constancy and precision required for individual dose determination. Reliance must still be placed upon after-the-fact estimation of doses presented by assessment of samples collected over the same periods as the volunteer exposures, from samplers located in immediate proximity to the exposure port of the aerosol-generating device.

Even were the problems of dose predictability resolved, suitability of an exposure method still remains dependent upon the purpose of the experiment. If the objective of a study is to determine whether or not man may be infected by an airborne agent in an essentially small-particle aerosol, the method employed by Gerone and his associates is quite appropriate. Similarly, the capacity of a virus to initiate disease in the lungs and tracheobronchial tree is susceptible to examination by this method, the artificially induced pneumonia and tracheobronchitis with strain 49882 HEK affirmatively answers such a question. Elucidation of the mechanisms of naturally acquired coxsackievirus A-21 infection and of the significance of particles of various sizes in natural transmission of disease is an altogether different matter.

Naturally occurring coxsackievirus A-21 illness

is an upper respiratory disease. Experimentally produced upper respiratory illness with this virus has been achieved by nasopharyngeal inoculation (3), by deposition of virus on selected sites in the upper respiratory tract, and by inhalation of large particle aerosols (4), which are primarily deposited in the upper respiratory tract.

In a series of experiments, Buckland and his associates circumvented the problem of precise location of deposition of airborne particulates by direct application of coxsackievirus A-21 to specific locations in the upper respiratory tract. Their findings showed the nasal mucosa to be exquisitely susceptible to infection, whereas the oropharynx and nasopharynx were refractory to doses several orders of magnitude greater. In subsequent studies, volunteers were infected with doses comparable to those directly instilled when presented in relatively large airborne particles, virtually all of which might be expected to be deposited on the nasal mucosa. These authors concluded that only particles retained in the upper respiratory tract are of significance in transmission of naturally occurring disease.

In attributing production of upper respiratory disease to the small particles generated with the Collison atomizer, Gerone and his associates have not rigorously excluded the contribution of that portion of the particles larger than 2 μ, which might be expected to be retained in the upper respiratory tract. From analysis of the particle size spectrum of the aerosol, approximately one-fifteenth the dose presented might be so retained (5). This may well be a significant quantity of virus, of itself capable of initiating infection.

Further experimentation, either by use of aerosols whose upper respiratory retention is negligible, or by bypassing the upper respiratory tract via an artificial airway, are needed if this matter is to be definitively resolved.

Most disappointing to this reviewer is the lack of information presented upon the airborne stability of coxsackievirus A-21 under varying conditions of relative humidity and temperature. The observations of Buckland and his associates indi-

cate a biological decay rate of 50% per min for virus in small particles and roughly 25% per min in the larger particles, if decay is linear. Such values are compatible with droplet infection. Far greater airborne stability is required for significant airborne transmission under ordinary conditions. Valuable information could be obtained by sequential examination of static aerosols with slit samplers or impingers.

In summary, the authors have described an aerosol used to induce infection in man. This discussant believes that further, more critical examination is required to definitively establish the significance of deep respiratory deposition of small particles in production of upper respiratory disease, and hence the appropriateness of the model for the study of naturally acquired infection. It is hoped that further studies will clarify this. Similarly, improvements in high-volume sampling, combined with knowlege of airborne stability of this virus, will permit more critical evaluation of the role of airborne dissemination in coxsackievirus A-21 upper respiratory disease.

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Author's Comments on the Discussion

P. J. GERONE, R. B. COUCH, R. G. DOUGLAS, AND V. KNIGHT

In Col. Gochenour's discussion of our paper, several points were made with which we are in complete agreement. Other issues were raised,

however, regarding which we would like to clarify the position or the conclusions that have been reached.

In regard to the large volume air sampler (LVS), the discussant has outlined its limitations as being (i) excessive evaporative loss of collecting fluid, (ii) inability to estimate particle size distribution, and (iii) its failure to "meaningfully quantitate airborne virus." The loss of fluid by evaporation in the LVS has not been a serious problem. When known concentrations of coxsackievirus A-?1 were added to the collecting medium and circulated through the LVS for periods three times longer than the sampling periods described in the paper, no virus loss occurred. On the contrary, when low-concentration aerosols were sampled, the reduced volume of fluid that had to be tested served as an advantage.

The LVS was not designed to measure particle sizes of the aerosols it samples; furthermore, no other equipment is available which can analyze particle size and, yet, handle these large volumes of air.

Under the conditions in which the LVS was tested, it was found to be a quantitative sampler. The data in the last figure of the paper were replotted (Fig. 1a) to show the relationship between virus concentration recovered and virus concentration in the room air. It is readily apparent not only that a relationship does exist, but that there is a direct proportionality between the amount of airborne virus in the room and the quantities recovered in the LVS.

The studies comparing the LVS and all glass impinger (AGI; Table 5) raised a question in the

TABLE 1a. *Coxsackievirus ,.21 aerosols used in volunteer inoculations*

Run no.	Dilution of inoculum pool	Suspension concn (log$_{10}$ TCID$_{50}$ per liter)	Aerosol concn (log$_{10}$ TCID$_{50}$ per liter)	
			Predicted	Actual
1	$10^{-4.8}$	8.0	1.74	2.08
2	$10^{-4.5}$	7.5	1.30	0.35
3	$10^{-4.5}$	7.5	1.30	1.24
4	$10^{-4.5}$	7.5	1.30	1.42
5	$10^{-4.5}$	7.5	1.30	1.42
6	$10^{-4.5}$	7.4	1.22	1.11

discussant's mind regarding the variability seen in virus recoveries and the consistency demonstrated in the fluorescein recoveries. It should be stated that two variables were present in the virus determinations which were not present in the fluorescein assays. These are: (i) the biological inactivation of the virus and (ii) the sensitivity of the cell cultures used in the assay procedure. The four virus recovery values with the LVS in actual TCID$_{50}$ ranged from 4.6 to 5.1 log$_{10}$, and are consistent with the 0.25 log$_{10}$ standard deviation of the assay procedure. This standard deviation, however, cannot be applied to the virus values obtained with the AGI, because those end points were calculated by the Fischer-Yates dilution technique. Despite the limitations imposed by this assay procedure, the mean recovery in the LVS and AGI were remarkably similar.

The disc ....rt also questioned the predictability of do.... .:dministered to volunteers with the experimental aerosols. We agree with his enumeration of the factors which may influence predictability. Undue emphasis, however, was placed un a few points which strayed from the line shown in Fig. 1 of the manuscript. The maximal deviation between the predicted and actual determination was 1.1 log$_{10}$, and only 3 of the 27 points plotted on the graph (1 in 9 determinations) were more than 0.5 log$_{10}$ from the predicted values. These results have been interpreted by the authors as representing good predictability for aerosol inoculations with *this virus*. This can be further supported by actual figures, shown in Table 1a, taken from the subsequent volunteer experiments. Five of the six predicted values were within 0.35 log$_{10}$ from the actual determinations and four of these were within 0.15 log$_{10}$ of the anticipated concentration.

The two main points that were established by the studies of sneezes and coughs were: (i) these expiratory events produce large numbers of small aerosol particles capable of remaining airborne for long periods of time, and (ii) sufficient quanti-
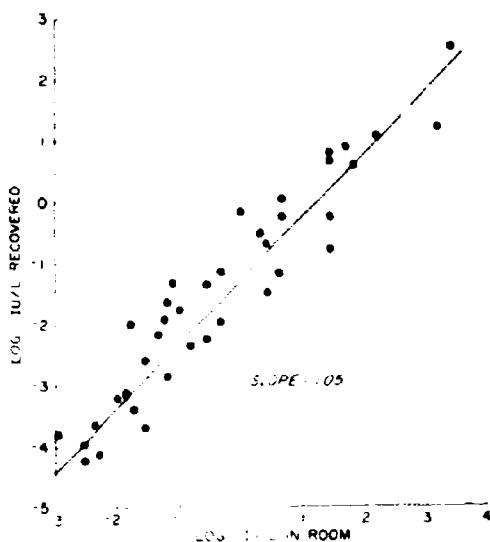
Fig. 1a. *Relationship of coxsackievirus A-21 concentrations in room air and quantities recovered by the large volume air sampler*

ties of coxsackievirus A-21 are present in these particles to induce infection in susceptible subjects. Additionally, it should be noted that most of the particles produced by sneezes and coughs are in a size range comparable to those generated by the Collison atomizer; however, because of the presence of small numbers of large particles in sneezes and coughs, the volume distributions of the natural and experimental aerosols are different. The distribution of virus according to number or volume of particles in natural aerosols has not been determined. The occurrence of airborne virus in cough specimens was found to be statistically related to the quantities of virus in the nasal and oral secretions (1).

The discussant's observation that the larger particles in the experimental aerosol may have initiated upper respiratory infection and illness in the volunteers may be valid, and was recognized by us in a previous report (2).

On the basis of the discussant's comments regarding types of clinical illness produced with coxsackievirus A-21 infections, additional clarification of our findings is necessary. The predominant clinical response produced by this virus is upper respiratory illness, regardless of whether the infection occurs in natural circumstances or follows experimental inoculation by nasal instillation, large-particle aerosols, or *small-particle* aerosols. With one strain (49889 HEK$_1$), however, lower respiratory illness was the predominant response and occurred only after small-particle aerosol inoculation. There appears to be no doubt that the upper respiratory passages are extremely susceptible to infection with this virus, and we agree that deposition at this site may be responsible for the consistent finding of upper respiratory illness in natural and experimentally induced disease. Finally, as stated in the previous paper (1), the question of how this virus is transmitted in nature has, at the present time, not been answered in this laboratory or elsewhere.

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Infection of Pigeons by Airborne Venezuelan Equine Encephalitis Virus

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INTRODUCTION

The experiments described were designed to provide information on the infectivity of Venezuelan equine encephalitis (VEE) virus for birds by the respiratory route. Laboratory studies by Chamberlain (2) showed that wild birds, including pigeons, could be infected with VEE virus by mosquito bite or by subcutaneous (sc) injection of virus. Overt signs of disease were absent in avian hosts, but viremia was produced for periods of 1 or 2 days, followed by the appearance of specific serum-neutralizing (SN) antibodies. The respiratory route of infection with VEE virus has been suggested previously. In noting that virus occurs in the nasopharyngeal washings of infected humans, Olitsky and Casals (10) recognized a potential for epidemics without insect vectors. Perhaps the most striking evidence of invasiveness of VEE virus by the respiratory route was provided by Slepushkin (11), who reported on infections in a large group of laboratory personnel after exposure to aerosols produced by breakage of a vial of virus. The susceptibility of birds to infections by the respiratory route had not been investigated. However, the possibility that the respiratory route was involved with arboviruses in nature was suggested by Holden (6) in studies with pheasants and eastern equine encephalitis (EEE) virus. Other cases of contact infection among birds by EEE and western equine encephalitis viruses were reviewed by Bourke (1).

COMPARATIVE SUSCEPTIBILITY OF FOWL TO AEROSOLS OF VEE VIRUS

The selection of an avian host for the subsequent studies of response to static aerosols of VEE virus was preceded by screening a number of species of fowl. Birds were exposed for 1 min to aerosols of the Trinidad strain of VEE virus. The

particle size distribution of the clouds was characterized by a mass median diameter of 1.5 to 2.5 μ and a slope of 3.5 probits per log diameter. Those values indicated that about 60% of the cloud mass was in particles between 1 and 3 μ in diameter. After exposure, the birds were isolated by dosage group in gas-tight cabinets and bled daily for viremia determinations. Serum neutralization tests were conducted on host sera collected before exposure and 21 days after exposure.

Included in the host range were leghorn chickens that had previously been shown to respond to an intravenous dose of <10 mouse intracerebral LD_{50} ($MICLD_{50}$) units. However, these birds, like ring-necked pheasants, hybrid chickens, and Peking ducks, were resistant to doses of 2,500 to 10,000 $MICLD_{50}$ inhaled. By contrast, White Carneau pigeons (3) proved susceptible. A viremic and serological response was obtained in 60 to 80% of the birds tested at an inhaled dose as low as 374 $MICLD_{50}$ (9). The marked cut-off in response below this level and high percentage infected with higher doses are illustrated in Table 1, with data taken from a number of experiments. All aerosol exposures were for 1 min only.

In general, viremic levels ranged as high as 10^9 $MICLD_{50}$ per ml of blood with no obvious dependence upon dose, once a minimal infective dose was given. The duration of viremia averaged 3 days, beginning on the 1st or 2nd day after exposure. VEE virus infections in pigeons, as in other birds (2), did not result in apparent illness or in histopathology. Such histopathological evidence of disease as did appear was usually ascribed by the pathologists to other causes, including agents of pneumonia, trichomoniasis, and bacterial meningitis, but even these were rare. During the course of these studies, no hypersensitivity was detected in previously exposed birds. Tests were

TABLE 1. *Response of White Carneau pigeons to respiratory doses of VEE virus presented in 1 min*[a]

Dose (MICLD₅₀ inhaled)	No. of birds viremic (positive/total)	Serological response (no. of birds showing positive SN indices/no. of birds tested)
3,770	4/5	4/5
2,291	6/9	8/9
1,960	5/8	4/6
1,349	5/8	5/7
589	5/6	3/3
374	7/8	6/7
135	0/8	0/6
76[b]	0/8	0/8
51[b]	0/5	0/5
Controls	0/24	0/24

[a] Aerosol conditions: 80% relative humidity at 80 F (26.7 C).

[b] Doses estimated by extrapolation from cloud concentrations at earlier cloud ages.

conducted for delayed skin reactions after injection of VEE virus antigen into the margin of the eye.

Almost without exception, the detection of viremia in pigeons over a 2-day period was followed by a significant rise in the titer of SN antibodies (>1.0 log increase in SN index). These same animals were resistant to respiratory challenge 3 weeks after the original exposure. Table 2 illustrates a typical set of responses to an initial and to a challenge dose of VEE virus. Although not shown, control studies in which nonresponders were later challenged indicated susceptibility indistinguishable from that of normal birds.

COMPARISON OF RESPONSES AFTER INHALATION AND INJECTION OF VEE VIRUS

The objective of one series of experiments was to compare the responses of pigeons to respiratory and subcutaneous doses of VEE virus (9). The respiratory dose was 1,349 MICLD₅₀ inhaled; the subcutaneous dose was 506 MICLD₅₀. The results of the experiment are summarized in Table 3. Viremic responses are presented as a function of day after dosage. Birds receiving virus by the respiratory route were not tested beyond day 4 because of previous data indicating that viremias normally terminated prior to that time. No data were available to indicate the duration of viremia after dosage by the sc route. Therefore, blood samples were collected and assessed for VEE virus on each of 10 successive days after injection.

Among eight pigeons receiving 1,349 MICLD₅₀ by the respiratory route, approximately 75% ex-

hibited viremias that first became evident in blood samples collected on the 1st or 2nd day after exposure. In the group injected with 506 MICLD₅₀ sc, viremias occurred in all eight birds, and virus was uniformly found in the blood on the 1st day after injection. However, the more rapid response of the injected birds was not obtained with a smaller dose. At a dose of about 5 MICLD₅₀, by the sc route, only 50% were found to be viremic, and this condition first occurred from 1 to 2 days after injection. Thus, as the minimal infective dose was approached by each route. the characteristics of viremias were indistinguishable.

The similarity of the serological responses after infection by each route is illustrated in Fig. 1. Apparently, once an infection was established, the rate and extent of appearance of SN antibodies was independent of the route by which the virus entered.

An additional criterion for the comparison of responses by each route of infection was the detection of virus in the cloaca and in the oral cavity. These tests were considered to be qualitative only because of the frequent occurrence of low titers that could not be reliably confirmed. However, with both the sc and respiratory groups, it was possible during the period of viremia to isolate and confirm the presence of VEE virus in the oral cavity, but not in the cloaca. Subsequent attempts to isolate virus on days 42, 43, 80, and 81 after infection was initiated were unsuccessful. These results add to the concept of a subclinical, but immunizing, type of infection.

BIRD-TO-BIRD TRANSMISSION OF VIRUS

It appeared logical to investigate cross-infections between birds, because both the data on respiratory susceptibility and the demonstration of virus in the oral cavity suggested the possibility of contact infections.

A device was fabricated to provide passage of air from viremic birds to normal animals (8). Two boxes of about 3-ft³ capacity were interconnected by a 3-inch duct through which air flowed at about 12 liters per min. To insure that arthropods would not pass from infected birds in one box to normal birds in a second, a 60-mesh screen was placed in the duct.

Six birds were infected by head exposure to static aerosols of VEE virus. These animals were placed in one box, and six normal birds were placed in the second. The birds remained in the enclosures for 3 weeks, except for short periods during the first 10 days when blood samples were collected daily. One of six normal birds developed specific viremia on days 9 and 10 of the 10-day test period. Considering that viremia and oral

TABLE 2. *Responses of White Carneau pigeons to V.E.E. virus administered by the respiratory route and subsequently challenged with virus by the same route[a]*

Dose inhaled with 95% confidence limits	Bird no.	Viremia (log $SMLD_{50}$ per ml of blood)					SN^b		Challenge dose ($SMLD_{50}$) with 95% confidence limits	Viremia (log $SMLD_{50}$ per ml of blood)				SN^b post-exposure
		1 day	2 days	3 days	4 days		Pre-exposure	Post-exposure		1 day	2 days	3 days	4 days	
3,715 inhaled in 1 min (2,344-5,888)	35	>3.5	>3.5	3.5	<1.5		0.1	2.0	3,379 inhaled in 1 min (2,361-4,781)	<1.5	<1.5	<1.5	<1.5	2.5
	28	<1.5	>3.5	>3.5	<1.5		0.6	2.0		<1.5	<1.5	<1.5	<1.5	1.8
	63	<1.5	>3.5	>3.5	<1.5		0.4	2.9		<1.5	<1.5	<1.5	<1.5	2.5
	40	<1.5	2.7	2.8	<1.5		-0.6	2.2		<1.5	<1.5	<1.5	<1.5	2.9
None (controls)	45	<1.5	<1.5	<1.5	<1.5		-0.2	0.4		3.3	3.1	2.3	<1.5	2.9
	38	<1.5	<1.5	<1.5	<1.5		1.0	0.7		2.9	2.9	2.5	<1.5	2.3
	34	<1.5	<1.5	<1.5	<1.5		-0.5	0.8		1.8	3.2	2.6	<1.5	2.2
	50	<1.5	<1.5	<1.5	<1.5		0.4	0.1		<1.5	3.5	2.5	<1.5	1.9
	62	<1.5	<1.5	<1.5	<1.5		0.5	0.1		<1.5	<1.5	2.4	3.0	1.1

[a] Aerosol conditions: 80% relative humidity at 80 F.

[b] Log units of virus neutralized.

[c] Birds were challenged 21 days after the original exposure.

TABLE 3 *Response of pigeons to respiratory and subcutaneous doses of VEE virus*

Day post-infection	Respiratory group[a]		Injected group[b]	
	Viremic (no. positive/total)	Confirmed oral swab isolate (no. positive/total)	Viremic (no. positive/total)	Confirmed oral swab isolate (no. positive/total)
1	3/8		8/8	
2	5/8		6/8	
3	5/8	1/8	6/8	
4	2/8		2/8	
5	No data		1/7	1/7
7-10	No data		0/7	

[a] The respiratory group inhaled 1,349 MICLD₅₀ in 1 min.

[b] The injected group received 506 MICLD₅₀ subcutaneously.

virus were apparent among exposed birds, the potential of cross-infection among pigeons appeared to be low. It might be noted that additional opportunities to detect cross-infections were afforded by placing normal animals in holding cabinets with viremic hosts. All such tests were negative.

EFFECT OF EXPOSURE TIME ON RESPONSE TO INFECTION

One possible explanation for the lack of cross-infections was an effect of exposure time, i.e., that dose-response data from a 1 - min exposure could not be extrapolated for the interpretation of effects when the same doses were given over extended periods of time. One might assume in this case that the passage of virus through the duct mechanism would not be in numbers equal to the minimal infective dose per minute that was noted in controlled aerosol trials. If, then, one were to postulate that infection does not occur unless a specific *rate* of exposure is achieved, regardless of the total dose presented, the lack of cross-infections could be explained. A system was developed (8) to permit exposure of a group of pigeons to aerosols at a dose rate less than the minimal infective dose per minute. With prolonged exposure, however, a total dose far in excess of the minimal infective dose could be inhaled. The scheme employed is illustrated in Fig. 2. After dissemination of virus, the aerosol chamber was mechanically purged for a period of 20 min. The remaining aerosol was then assessed, and subsequently was allowed to undergo biological decay until the concentration approached the minimal level for estimation of viral content. At this time, the aerosol was again sampled for esti-

mation of viral concentration. Further cloud aging occurred to the extent necessary to yield desired doses. The level of infective virus during exposures was estimated by extrapolation from the line established by the two assays. Justification for the procedure was given by earlier work which indicated that biological decay was linear, and respiratory infectivity of VEE virus, for guinea pigs, was consistent over the cloud ages of interest to this study.

The results of five experiments in which birds were exposed to aerosols for extended periods of time are presented in Table 4. Periods of exposure, total inhaled doses, and doses in the first minute of exposure were varied. In four of five experiments, birds were given total doses over periods of 25 to 180 min that far exceeded the infective doses discussed previously for 1-min exposures. In only one experiment did pigeons generally respond with viremia and production of SN antibodies. In that test, 6,037 MICLD₅₀ were inhaled over 60 min, but, more important, the dose in the 1st min of exposure was 304 MICLD₅₀ inhaled. This value was within the minimal infective dose range established in 1-min exposure trials. Note that in the test where the 1st min dose was 124 MICLD₅₀ inhaled, about three times the minimal infective dose was accumulated in the first 5 min of exposure. By the end of 60 min in that trial, 10 to 20
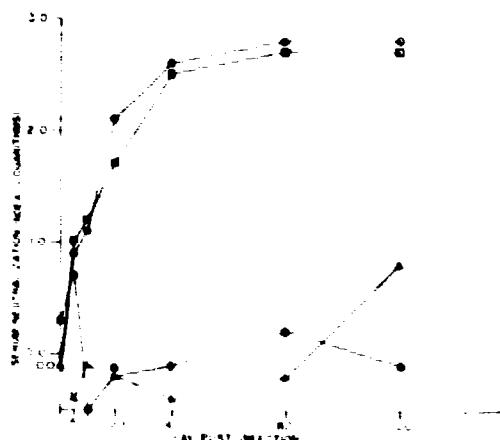
FIG. 1. *Results of neutralization tests against Venezuelan equine encephalitis virus by sera collected from White Carneau pigeons at various periods after injection. Symbols:* ○ = *birds developed viremia after respiratory dosage;* △ = *birds did not develop viremia after respiratory dosage;* □ = *birds developed viremia after dosage by the subcutaneous route;* ● = *birds served as environmental controls and were not viremic. The open square and circle to the left of the vertical axis should be read as being superimposed on the triangle on that axis.*

times the minimal infective dose had been accumulated, and yet viremias and SN antibodies did not occur. It thus appeared from these data that infection was dependent upon rate of exposure and not total dose.

It was of interest to test pigeons for effects of extended exposure in terms of response to subsequent challenge. Accordingly, the dose-response curve was re-estimated by exposure of birds that had not shown viremia or neutralizing antibody formation after exposure to a total dose of 2,934 MICLD₅₀ inhaled over a 60-min period. The results indicated that the previous experience with virus had no detectable influence on the subsequent disease response to infective doses. Birds were viremic after an inhaled dose of 589 MICLD₅₀ and, as expected, failed to respond to 19 MICLD₅₀.

EFFECT OF ANTIMICROBIAL DRUGS ON SUSCEPTIBILITY

It is of interest, both epidemiologically and academically, to detect mechanisms that alter the normal dose-response relationship of virus and

FIG. 2. *Aerosol concentration of Venezuelan equine encephalitis virus as a function of age. Concentrations are in terms of doses per minute for White Carneau pigeons expressed as MICLD₅₀ units. The open circles indicate aerosol concentrations estimated by sampling. The solid line indicates the periods of natural cloud total decay; the dotted line indicates a period of mechanical purging. The hatched area illustrates the dosage inhaled by pigeons during a 60-min exposure. Bands above and below the open circles indicate 95% confidence limits.*

TABLE 4. *Responses of White Carneau pigeons to respiratory doses of VEE virus presented over extended periods*[a]

Total dose (MICLD₅₀) inhaled	Maximal dose per min	No. viremic (no. positive/ total)	Serological response (positive/ total)
1.7 in 60 min	Minute	0/5	0/4
1,236 in 180 min	51	1/5	0/4
2,934 in 60 min	22	2/15	2/15
4,544 in 60 min	124	0/8	0/6
6,037 in 25 min	304	5/7	5/7

[a] Aerosol conditions: 80% relative humidity at 80 F.

host. More specifically, it was of interest to detect factors which altered the rate process indicated above. Such a mechanism was suspected when a group of birds responded with viremia and formation of neutralizing antibodies to what appeared to be an abnormally low dose. This group had been given a supplement of Coaa-terramycin (oxytetracycline with glucosamine and vitamins; Chas. Pfizer and Co., Inc., New York, N.Y.) and HepZide (nithiazide; Merck and Co., Inc., Rahway, N.J.) in the feed for 2 weeks prior to the test. To test for a possible relationship between the drugs and susceptibility to VEE virus, 15 birds were held for 2 weeks without supplement, 10 birds received Coaa-terramycin in the drinking water at a dosage of 400 mg per gal of water, and 5 birds received Coaa-terramycin plus HepZide at a dosage of 800 mg per gal for 2 weeks. All birds were then exposed to a total inhaled dose of 2,934 MICLD₅₀ units over a 60-min period. The highest dose per min was 22 MICLD₅₀ units inhaled, or about one-tenth the usual infective dose. Of the untreated birds, 13% developed viremia and neutralizing antibodies, 40% of the Coaa-terramycin-treated birds responded, and 60% of the birds receiving Coaa-terramycin plus HepZide responded. The results of a second test in which oxytetracycline alone was included in the drinking water (200 mg per gal) of birds for 2 weeks prior to exposure to VEE virus aerosols gave different results. There was no viremic response among eight birds to total doses of 668 MICLD₅₀ inhaled over 60 min with 1.4-min doses of 27 MICLD₅₀ inhaled. In this case, susceptibility was not increased by antibiotic treatment. It is possible that the effects noted here are complex and will require extensive investigation for a full definition.

CONCLUSIONS AND DISCUSSION

The principal findings of these studies may be summarized as follows.

(i) VEE virus can infect avian hosts through the lower respiratory tract, although marked species differences occur. The minimal infective dose for White Carneau pigeons was between 135 and 374 MICLD$_{50}$ units inhaled in not more than a 1-min period. Infection was characterized by viremia over a 2- to 3-day period, virus in the oral cavity during the viremic period, and production of neutralizing and protective antibodies. Far higher concentrations of virus were usually tolerated without viremic or serological response if inhaled at a rate less than 374 MICLD$_{50}$ per min. The full potential of this resistance mechanism in terms of duration of effectiveness is not known. This phenomenon, however, is possibly involved in natural resistance to cross-infections between birds. Further, the possible implication of the rate process for successful aerogenic immunization should not be overlooked.

(ii) Treatment of pigeons with Coss-terramycin and HepZide, or Coss-terramycin alone for 2 weeks prior to exposure to viral aerosols altered a normal resistance mechanism associated with respiratory challenge. With such treatment, birds became susceptible to a dosage rate 1 log lower than that normally seen.

(iii) Subcutaneous injection of 5 MICLD$_{50}$ of virus into pigeons resulted in infections which could not be distinguished from those which followed respiratory exposure to 374 MICLD$_{50}$ inhaled. Comparisons were based on level and duration of viremia, level and duration of neutralizing antibodies, and occurrence of virus in the oral cavity. In view of the similarity of responses, it is reasonable to assume that the sites of infection were the same regardless of route. Thus, the difference in minimal infective doses was not a function of requirements of the infection sites, but, rather, a function of factors which inhibit arrival at such sites.

A fraction of the difference was due to incomplete retention in the respiratory system. On the basis of data presented by Hatch and Gross (5) for mammals, the particle size range employed in these studies would yield retention of 25 to 50%, of inhaled particles in the lower respiratory system or about 100 to 200 MICLD$_{50}$. The precise amount of inhaled dose which was retained, however, is not known. Hatch and Gross point out a number of factors that affect retention of aerosol particles, including tidal volume, breathing frequency, particle size, and species of host. These variables have been controlled to the maximal extent in our aerosol studies to permit valid, though relative, estimates of treatment relationships. The precise effects of each factor on retention in pigeons are not known, however, and thus

comparisons of responses by route have limitations.

A component of the remaining difference in effective doses by the two routes would appear to be nonspecific resistance associated with the respiratory system. Resistance of the pigeon against VEE virus by the respiratory route was considered from the standpoint of virus-induced and host-induced mechanisms. In the case of the former, autointerference was a distinct possibility because of the test procedures. Where graded doses, as in dose-response studies or in extended exposure time trials, are achieved by cloud aging, the aerosols will contain increasing proportions of dead virus. Thus, effects of decreasing dosages which might be ascribed to the host could be due to interference, because the percentage of inactive virus increases with decreasing amounts of active virus. This was not a problem, however, as shown by a study in which birds were given a large dose of inactive virus in aerosol form. Following this procedure, 1-min exposures were made to viral aerosols at two concentrations for dose-response estimation. The birds responded to 513 but not to 19 MICLD$_{50}$ inhaled, suggesting that inactive virus was ineffective in preventing infection.

The host-associated mechanisms of nonspecific resistance are not known, but might include phagocytosis, antiviral substances present in the lower respiratory tract (4), or physical removal by the proteinaceous fluid film of the alveolar membrane and the mucous blanket which begins in the respiratory bronchioles (5). Whatever the system, it must be compatible with the rapid rate of viral inhibition indicated in these experiments.

The mechanism associated with the reduction of the nonspecific resistance rate by drugs is also not clear. One may postulate direct antagonism of the drugs towards a protective substance or mechanism, or a withdrawal of a substance or mechanism due to the presence of effective drugs. One interesting possibility is that the antibiotic may eliminate gram-negative endotoxin producers of the intestinal tract. According to Ho (7) and Stinebring and Youngner (12), endotoxins induce the formation of interferon or cause the release of preformed interferon in rabbits and mice, and thus may indirectly affect general resistance of the host to viruses. A similar and additive effect could be attributed to nithiazide in those birds in which trichomonads existed and possibly stimulated nonspecific viral inhibitors. In brief, the drugs employed may have eliminated organisms or their products which induced or released active interferon. This proposal is purely speculative, however, and must be examined experimentally.

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Discussion

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Dr. Miller has presented some intriguing observations on the response of white Carneau pigeons to airborne Venezuelan equine encephalitis (VEE) virus.

Infection by subcutaneous and by respiratory inoculation was benign. Not surprising are the relatively brief viremias, prompt antibody responses, and resistance to reinfection.

Despite demonstration of virus in the oral cavity of infected birds, airborne bird-to-bird transmission was demonstrated to be a rare occurrence, perhaps best explained by the size of the minimal airborne infecting dose and the poor aerosol-generating capacity of the bird itself.

The response of the pigeons to graded acute doses of airborne VEE is quite unlike that of mammals exposed to the virulent virus. It does resemble, in some respects, the responses of mice and of monkeys to airborne attenuated VEE (1). In rhesus monkeys, an abrupt threshold of infection is manifest at approximately 1,000 guinea pig intraperitoneal 50% infectious doses (GPIPID$_{50}$), with no infections occurring below this point, and consistent infection above this level. This, to a degree, is comparable to the abrupt cutoff in the pigeons at a level of approximately 374 MICLD$_{50}$. On the other hand, the continuing partial response in groups of birds at doses ranging up to 10 times this dose is remarkably similar to the partial response of mice over a 2-log range of exposure to the attenuated virus.

To me, the most intriguing observations reported are the resistance of the pigeons to infections when exposed to large doses of virus presented at rates less than one ID$_{50}$ per minute. No parallel in mammals is known to the author. Indeed, in mice exposed to virulent VEE at the rate of 20 MICLD$_{50}$ per minute (2), the respiratory LD$_{50}$ was 27 MICLD$_{50}$ presented, a value in consonance with those obtained in short exposure times.

Additional data obtained by Miller, but not presented in his paper, substantiate the validity of extrapolation of the linear decay of VEE in the system used. It does not appear reasonable to challenge the validity of the dose estimation in these studies.

A slightly different type of experiment might eliminate some factors inherent in the studies described. The role of decaying, as against dead, virus might be eliminated from consideration if the doses were presented at the same rates with a

dynamic cloud by use of a modified Henderson apparatus, as was done in the mouse experiment cited. Further, such a system would permit larger volumes of air to be sampled and, hence, direct estimation of the dose presented rather than extrapolation.

It is desirable to extend these studies of the significance of dose rate to other hosts and to other airborne infections wherein the minimal acute respiratory dose is a value larger than 10 organisms presented. Miller has added yet another variable, dose rate, to be entered into

the complex equation describing host agent interaction in airborne infection.

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Physiological Responses of Airborne Bacteria to Shifts in Relative Humidity

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Most information currently available on the behavior of airborne cells has been collected by investigators studying aerosols held in static environments Wells and Riley (15), for example, showed that survival of bacteria was markedly influenced by humidity and temperature, and that the effects varied between bacterial species. Death of airborne bacteria has been observed to increase with a rise in humidity (2, 17), but contrary findings have also been reported (6, 16). Maximal death rates were found between 50 and 60% relative humidity (RH), and death has been reported to occur at more than one rate (5). There are more recent reports of multiple-stage death rates (12, 13, 14).

Few reports have been published, however, describing possible experimental techniques for subjecting microorganisms in air to shifts in RH, although it is well understood that such shifts do occur in natural airborne environments. Brown (1) regulated moisture in static chambers with salt solutions and sprayed water to produce intermediate changes. Hemmes (9) reported similar experiments with shifts in RH produced by spraying water into the aerosol chamber. One may also effectively produce limited rehydration of airborne particles by permitting the incoming air of the atomizer to be at a higher humidity level than that of the final humidity condition (12). The principle of the adiabatic expansion of a gas has been used successfully by Druett (*personal communication*), who found that a rapid decrease in viability occurred if the expansion raised the humidity sufficiently to cause moisture condensation onto the particles. No effect was observed at low relative humidities. Other unreported experiments have apparently been performed, as discussed by Wolfe (cited in 8), wherein pressure changes were produced within an aerosol chamber. A change in temperature would effect a change in RH, although the task of ascribing noted biological effects to humidity alone would be difficult.

Our purpose in studying the effects of sudden shifts in RH on airborne bacteria already equilibrated to one humidity condition was twofold: first, we were interested in applying laboratory findings to natural environments where temperature and humidity are constantly changing; and second, we were interested in possible death mechanisms—noting effects of shifts on subsequent biological behavior might furnish us with additional clues to such mechanisms. It is the purpose of this paper to report our findings and to discuss some of the implications of our results.

DUAL AEROSOL TRANSPORT APPARATUS

We achieved an abrupt shift of humidity in an air stream by diluting it with a second air stream at a different humidity; air temperature was held constant at 21 C. Only a brief description of the equipment and methodology will be made, since details have been previously reported (8). A 45-ft (13.7-meter) duct, 6 inches (15.2 cm) in diameter, was inserted 2 ft (61 cm) into another 45-ft duct, 8 inches (20.3 cm) in diameter. Each duct was equipped with numerous sampling ports. The point of juncture, where mixing of two air streams occurred, was called the confluence point. Linear air flow through both ducts was equal, the transit time per duct was about 5.7 min; total aerosol time was about 11.3 min. The calculated dilution of the primary air stream at the confluence point was 0.56, or approximately 50%. Humidity could either be increased or decreased by the dilution effect Figures 1 and 2 illustrate the apparatus

Bacteria were sprayed into the primary air stream Concentration of particulate matter was
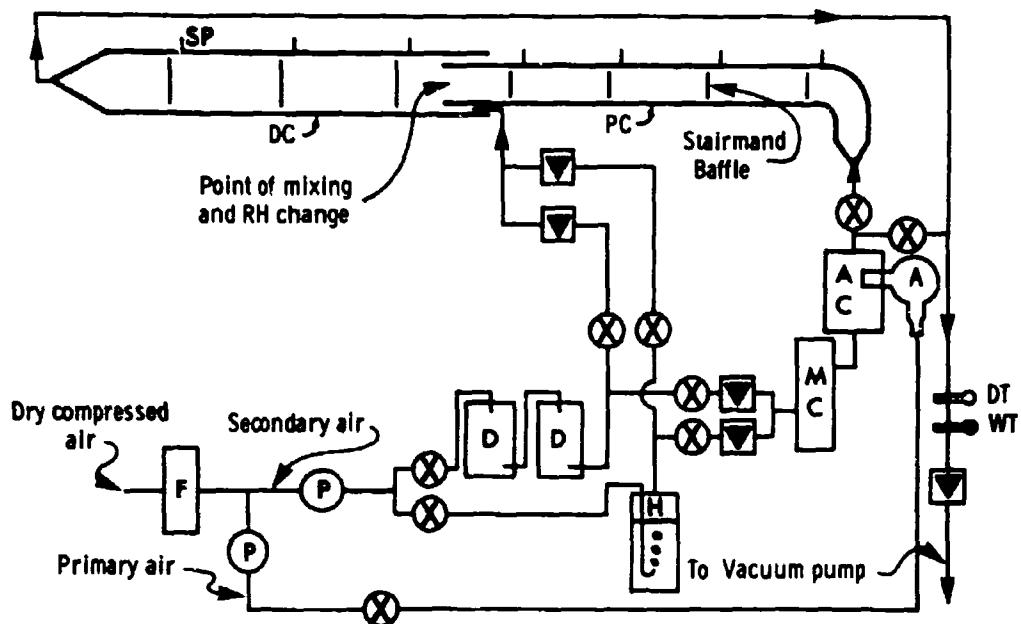
FIG. 1. *Schematic diagram of the dynamic aerosol transport apparatus. PC, primary aerosol chamber; DC, diluted aerosol chamber; A, reflux-type Wells' atomizer; AC, atomization chamber; MC, mixing chamber for conditioning of air; D, Lectrodryer; P, pressure regulators; F, filter; SP, sampling ports; H, humidifying chamber; DT, dry-bulb thermometer; WT, wet-bulb thermometer; ⊗, valve; ▼, rotameter.*

FIG. 2. *Lateral view of the dynamic aerosol transport apparatus and ancillary equipment PC, primary aerosol chamber; DC, diluted aerosol chamber; SP, sampling port; CP, control panel; LA, light scatter apparatus.*

measured by forward-angle light scatter, and biological assay was made on samples collected either by slit samplers or impingers. Unless noted otherwise, we cooled 21.5-hr cultures to 4 C and then sprayed them from a refluxing atomizer, wherein the temperature usually increased to 15 C.

Figure 3 shows a hypothetical example, typical of observed results, for the purpose of defining parameters. The physical decay always followed first-order kinetics. The measured concentration of particles in the primary air stream immediately before dilution, compared with that in the secondary stream immediately after dilution (i.e., the apparent dilution ratio, ADR), was usually slightly higher than 0.56; sections A and B of Table 1 list some observed mean ADR values. Analysis of variance of three sets, 20 runs for each condition of shift-up, shift-down, or no shift, and disregarding other variables, indicated a 95% confidence interval of ±0.01 for all sets; differences between these three sets exceeded the 0.1% level of significance. These data were interpreted as indicating that the particles either increased or decreased in volume as a function of shift in RH. For example, if particles decrease in size, they scatter less light; therefore, the apparent dilution seems to increase and the ADR becomes smaller than without a shift in RH, and vice versa.

Since physical decay in the duct system was small and consistent, we refer to the sum of physical and biological loss as biological decay. The latter was always greater than physical decay and, in the primary air stream, usually followed first-order kinetics: "tailing" sometimes occurred after a humidity shift, but for comparative purposes we assumed first-order kinetics in all instances. Usually the biological loss, or biological dilution ratio (BDR), as a result of dilution at the confluence point, corresponded to the ADR (Fig. 3); important exceptions are noted below. Biological loss observed under the final conditions, as compared with loss under the initial condition, was defined as the dynamic-humidity-death (DHD) ratio (Fig. 3). If no change occurred, the theoretical ratio was 1.00; less than this number indicated enhanced death, and a number larger than 1.00 indicated that death process had decreased as a result of the shift. The mean DHD of 21 aerosols subjected to no shift in RH was 1.04 with a 95% confidence interval of ±0.05. We assume from this that DHD ratios greater than 1.10 or less than 0.90 are significant.

Serratia marcescens grown in, and sprayed from, dilute Trypticase Soy Broth (BBL) evinced an increased death rate (sorbed death, 11) when the RH was shifted from low to high values, but this effect was decreased if cells were sprayed
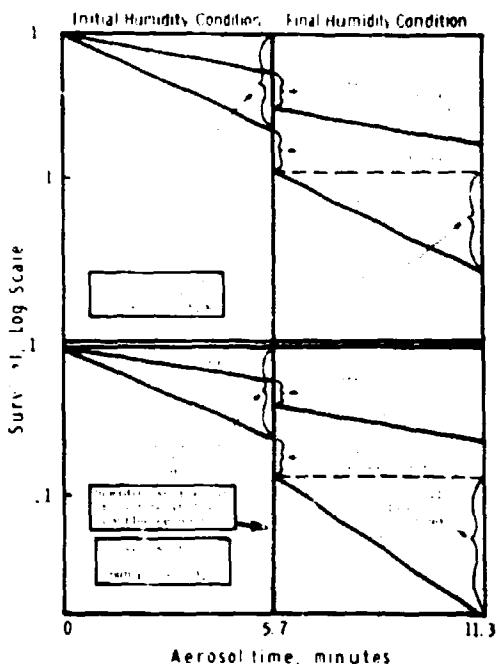
FIG. 3. *Theoretical behavior of bacteria aerosolized in the Dual Aerosol Transport Apparatus. There are two distinct intervals of both biological and physical loss, and there is loss caused by dilution. The theoretical dilution ratio, based on the geometry of the system, is 0.56. The apparent dilution ratio (ADR), measured by light-scatter, was approximately 0.59. A shift up in humidity increased the ADR, whereas a shift down decreased it. If no biological loss occurred at the confluence point, then the biological dilution ratio (BDR) was approximately equal to the ADR. We have frequently observed the BDR to be as much as 10 times the ADR. The initial biological loss divided by the final biological loss has been defined as the dynamic-humidity-death (DHD) ratio. In the upper example, with no change in humidity, the DHD ratio is approximately 1.00. In the lower, hypothetical example, where the change in humidity is unspecified, the DHD ratio shown is 0.77, indicating that the change was detrimental to survival. We have occasionally observed DHD ratios greater than 1.00.*

from a temperature-controlled, nonrefluxing (TCNR) atomizer at 4 C (section A, Table 1).

No sorbed death was noted (section B, Table 1) when *S. marcescens* was grown and sprayed in a chemically defined medium (3). Initially, there appeared to be a "toxic" effect of dilute Trypticase Soy Broth, because the DHD ratio was low (section C, Table 1) when cells were grown in chemically defined medium and resuspended in dilute Trypticase Soy Broth medium. It is of interest to note, however, that sorbed death was eliminated at humidity values above

TABLE 1. *Summary of results obtained when airborne bacteria were subjected to shifts in relative humidity*

Pertinent test conditions[a]	Per cent relative humidity conditions		ADR or BDR[b]	DHD ratio[b]
	Initial	Final		
Serratia marcescens				
(A) Grown and sprayed in dilute Trypticase Soy Broth (DTS)	24	51	ADR 0.61	0.71
	50	50	ADR 0.59	0.97
	24	47[c]		0.89
	90	57	ADR 0.48	1.10
(B) Grown and sprayed in chemically defined medium (CDM)	27	37	ADR 0.60	1.10
	51	51	ADR 0.57	1.08
	93	72	ADR 0.52	1.10
(C) Grown in CDM, resuspended and sprayed in DTS	22	54		0.62
	59	72		1.00
	24	55[c]		1.00
Atomizer fluid at 21 C	25[c]	53		— x[e]
Atomizer at 21 C with 1 mg/ml of chloramphenicol	25[c]	53		0.91
(D) Grown in DTS, resuspended and sprayed in CDM	25	52		0.90
(E) Grown in CDM and stored at 4 C for noted times, then resuspended in DTS at 4 C for 30 min and sprayed			Initial loss[b]	
(0 hr)	22[d]	52	71[d]	0.75
(1 hr)	22[d]	52	50[d]	0.86
(5½ hr)	22[d]	52	46[d]	1.20
(7 hr)	22[d]	52	21[d]	0.53
Pasteurella pestis A1122				
(F) Grown in Heart Infusion Broth	28	46	BDR 0.28	0.44
	28	28	BDR 0.59	1.00
	39	26	BDR 0.70	3.40
	87	61	BDR 0.16	1.00

[a] Refluxing atomizer; fluid chilled to 4 C before spraying, unless otherwise noted.

[b] See Fig. 3.

[c] Sprayed with modified, nonrefluxing atomizer with temperature control at 4 C.

[d] Single experiment; all other data are mean value of three or more aerosols.

[e] No survivors after shift in humidity.

59% RH, and when cells were sprayed from the TCNR atomizer at 4 C, the DHD ratio was 1.00. If cells were grown in dilute Trypticase Soy Broth and sprayed from chemically defined medium (section D, Table 1), the DHD ratio was higher than above; hence, dilute Trypticase Soy Broth was not toxic. Further evidence for nontoxicity is shown in section C, Table 1; cells grown in chemically defined medium and sprayed from dilute Trypticase Soy Broth at room temperature were so sensitive to sorbed death that no viable cells were recovered from the second duct, but the addition of 1 mg/ml of chloramphenicol to a similar suspension practically eliminated the detrimental effects of sorbed water.

The latter effect might be considered a protective one, but a similar result was obtained when cells were grown in chemically defined medium and stored at 4 C for various times before being added to dilute Trypticase Soy Broth to be sprayed (section E, Table 1). Storage in the cold for 5.5 hr changed the DHD ratio from 0.75 to 1.20; additional storage caused the DHD ratio to decrease to 0.53. This decrease was more apparent than real, however, because of the marked change in initial loss that occurred as a result of the cold storage period (see Table 1); the final biological decay, as a result of the 7-hr storage period, was less than that of the final decay of cells stored for 5.5 hr and equivalent to the final

decay of cells grown and sprayed in chemically defined medium.

Pasteurella pestis A1122 was grown and sprayed in Heart Infusion Broth (Difco). Although this species exhibited sorbed death similar to *S. marcescens*, there were three distinct differences between the species (section F, Table 1): (i) there was a change in death rate as a result of a shift-down in RH, a phenomenon we never observed with *S. marcescens*; (ii) the DHD ratio as a result of this change was 3.40, i.e., the death rate decreased markedly rather than increasing, (iii) instantaneous death (i.e., the rate was too rapid to measure) often occurred after humidity shifts (note last BDR, section F, Table 1). Also, preliminary evidence indicated instances of dissonance (3), or dilution shock, and a dependence on both constituents in, and temperature of, the sampling medium. For example, in one experiment in which plates, before being incubated, were chilled for 2 hr after the sample from an aerosol was inoculated, a twofold increase in colony numbers was found over the number on plates incubated immediately. In the same experiment, the addition of 1% whole blood to the medium caused a fourfold increase in colony numbers. Whole blood did not increase the number of colonies produced by unstressed populations. The actual extent of these increases varied with aerosol age.

APPLIED INTERPRETATIONS

These findings show that changes in RH do influence subsequent survival of airborne bacteria. The evidence indicates that this effect might be applied to air-sterilization processes. For example, air conditioning equipment might be cycled to lower the air contamination of public places, such as hospitals, schools, institutions, etc. (5). The study has not indicated specific or generally applicable RH changes, or rates of changes, that might be most lethal, nor is there direct evidence that survival after a shift in RH is different than it would have been in the second condition without a change. There is, however, presumptive evidence for this, in that we never observed *S. marcescens* cells to die as rapidly at 53% RH as they did when shifted from 25 to 53% RH (section C, Table 1). The spray temperature in this instance was more equivalent to natural conditions (21 C) than in other experiments (4 to 15 C). Further, little more than 10% of airborne *P. pestis* cells survived a shift-down in humidity from 87 to 61% RH, although the death rates before and after the shift were observed to be identical (section F, Table 1).

The difficulty of finding interpretations mean-

ingful to natural situations lies in the obvious dependence of airborne behavior on the history of environment of the culture before aerosolization. Goodlow and Leonard (7) previously pointed out the importance of such conditions. Rigid standardization in an effort to attain replicability does not aid the interpretation, because we usually do not know the cultural history of bacteria found in nature.

THEORETICAL INTERPRETATIONS

There are additional difficulties influencing our attempts to interpret these data from a theoretical viewpoint. We can justifiably point out some previously suspected mechanisms that either are not applicable or act only indirectly. Dehydration alone does not kill cells, otherwise freeze-dried cells would not survive as they do (4). Moreover, Hess (10), in a most important contribution, showed that little or no loss of viability occurred at any RH tested if cells were held airborne in oxygen-free chambers. Our data show that rehydration can cause death; interpreted broadly, these facts imply that part of the observed death may be caused by the act of sampling. In some instances, additional colonies arose when sampling plates were cooled before they were incubated or when nutrients not required by unstressed cells were added. Since cells ruptured by osmotic shock are unlikely to repair such damage, osmotic shock cannot be solely responsible for death. In fact, we may reasonably suggest that no currently used assay is accurate for cells injured by aerosolization.

From these data we theorize that airborne cells may be metabolically active. Substances such as chloramphenicol, or conditions such as low temperature that decrease metabolic functions, tend to increase survival capacity. Dehydration undoubtedly decreases metabolic functions, but probably in a manner that leads to an imbalanced but slowly readjustable condition. Cells sampled before readjustment die as a result of further imbalance unless provided with a situation where additional slow change, or repair, can take place. Cells normally exist in a variety of "states" because of the division cycle and differences in microenvironments. Therefore, individual cellular responsiveness to aerosolization ought to vary, and this is apparently what happened in our studies.

The evidence indicates that no single structural injury (e.g., hydrogen bond breakage, deoxyribonucleic acid denaturation) can account for all death observed and that measured behavior (colony formation) is highly dependent on functional activities of the cell

SUMMARY

In summary, the aerobiologist places a biological system, the bacteria, in a hostile and ill-defined environment, the atmosphere, for the purpose of studying air-bacterium interactions. Measurement of this interaction is in terms of survival. Survival has been shown to depend not only on physicochemical reactions of the somatic, structural components of the cell, but also on those functional, physiological, dynamic properties of all living systems, termed adaptability or responsiveness. The problem, whether one is assaying infectivity or is searching for clues pertinent to death mechanisms, is to separate the two effects.

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Discussion

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In the experiments which Dr. Hatch has described, the immediate effect of an abrupt change in relative humidity on an airborne microorganism was expressed as a deviation from the expected reduction in aerosol concentration due to the dilution by the additional air introduced at the confluence point. The biological dilution ratio, based on samples, was compared with the apparent dilution ratio based upon light scatter measurements. The biological loss observed during the 5.7-min aerosol transit time in the second half of the apparatus was compared with the equivalent loss observed in the first half, and was expressed as the dynamic humidity death ratio.

Regarding the immediate effects of an abrupt change in relative humidity on airborne microorganisms, one might suggest that not only are the effects dependent upon the direction and magnitude of the change but, perhaps, also upon the

rate of change of relative humidity. If, in the apparatus described, one assumes that the aerosol from the first tube mixes perfectly with the additional air introduced at the confluence point and that temperature is constant throughout, one wonders what time is required to achieve uniform relative humidity in the mixed aerosol beyond the confluence point. With adequate mixing, the equilibration time is probably rather short and dependent upon the diffusion rate of water vapor. One could perhaps assume that the small airborne particles containing microorganisms come to equilibrium with their micro-environment at a rate greater than that at which the environment is changing. Undoubtedly the equilibration rate of the airborne microorganisms with their environment would be influenced by the nature of the material in the particle deposited by evaporation of the suspending fluid from which the microorganisms were originally atomized. Other factors such as strain of a given species and the age of a culture and its metabolic state, as influenced by temperature or chemical composition of the suspending fluid, also have been shown to affect the behavior of airborne microorganisms subjected to an additional stress such as a change in relative humidity.

A differing biological loss observed during the initial and final 5.7-min aerosol transit periods was identified by Dr. Hatch as the dynamic humidity death ratio and was based upon the assumption that first order kinetics were followed during the initial and final aerosol transit periods. Assuming that a simple exponential decay does occur, one could as readily express the biological loss as a decay rate, which could perhaps be useful in predicting biological loss for time periods other than those obtained in this apparatus. In addition, by computing decay rates, one could separate the physical and total loss, as measured by light scatter and sampling, respectively, to obtain a true biological decay rate. In using light scatter measurements to indicate particulate concentration of an aerosol, one must be aware of the fact that the light scattered from a sample of the aerosol is not restricted to particles carrying microorganisms.

The employment of a mixed aerosol containing the test organism and a tracer such as *Bacillus subtilis* spores is suggested, since, from the test organism-tracer ratio, one can obtain viability data independent of sampler efficiency and the extent of aerosol dilution. To eliminate the influence of a possible biological loss of the tracer, one could employ radioactively tagged microorganisms as a nonviable tracer.

Such tracer techniques would also be of assistance in elucidating the "tailing" or deviations from an exponential decay rate which have sometimes been observed after a change in relative humidity.

Effect of Nitrogen Dioxide on Resistance to Respiratory Infection

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INTRODUCTION

In studies of the effect of atmospheric pollutants on health, the basic aspects that must be considered are: the direct damage due to acute and chronic exposure, the role of a pre-existing disease on susceptibility to acute and chronic exposures, and the effects of acute and chronic exposures on resistance to secondary stresses such as respiratory infection.

Air pollutants exert their effect by contact between the pollutant and the body, normally at the surface of skin and exposed membranes. The extent of damage is related to the pollutant's physicochemical properties, its concentration, and the duration of exposure.

For example, among the physicochemical properties, solubility is important. The part of the respiratory system upon which a pollutant may act depends on solubility. A gas of low solubility, such as nitrogen dioxide, penetrates into the lower respiratory tract and exerts its effect in this portion of the respiratory system.

The severity of the tissue response is usually the product of the concentration of the pollutant and the duration of the exposure. Although very low concentrations can sometimes be inhaled for long periods of time without causing any observable effects, inhalation of the same total amount of the gas over a short period of time or as a single breath can result in severe tissue damage and toxic response (14).

The effects of gaseous air pollutants on the membraneous surfaces of the respiratory system are of special interest from the standpoint of resistance to respiratory infection. An irritant gas reaching the epithelium of the trachea or the bronchi can paralyze cilia, alter mucus flow, affect phagocytic activity, and in severe exposures destroy the surface layers of the epithelial lining. These functions constitute the major defense mechanisms and play an important role in respiratory infections.

Nitrogen dioxide is one of the most abundant atmospheric contaminants in many communities. It is emitted in large quantities in the exhausts of automotive engines and is a by-product of natural gas combustion (26). In recent years, it has been increasingly recognized that exposure to oxides of nitrogen (nitrogen dioxide and nitric oxide) can occur in a wide variety of situations. Dangerous accumulations of nitric oxide and nitrogen dioxide can occur, for example, in agricultural silos (17), in enclosed mineshafts after detonation of explosives (3), and in industrial processes requiring the handling of nitric acid (7) A time interval of a few hours after acute exposure usually elapses before symptoms develop (20). After this interval, acute pulmonary edema, cyanosis, severe dyspnea, and bronchopneumonia characteristically develop. When not immediately fatal, the acute episode may be followed by the development of bronchiolitis obliterans, which may cause death during the next few weeks (17) or may lead to persistent abnormalities in airflow.

In the past, the effect of air pollutants on resistance to infection has been studied from two viewpoints, namely, epidemiology and animal experimentation. The discussion in this paper will be limited primarily to the effect of acute and

chronic exposures to the air pollutant nitrogen dioxide on resistance to infection produced by respiratory challenge with airborne *Klebsiella pneumoniae*.

EFFECT OF ACUTE EXPOSURE

The methods used for acute exposure of experimental animals to nitrogen dioxide and for respiratory challenge with aerosols of *K. pneumoniae* have been described in detail in previous publications (21, 22).

Briefly, *K. pneumoniae* type A, strain A-D, was used. It was isolated on Blood Agar Base (Difco) from the heart of an intraperitoneally injected mouse. Stock cultures were prepared on Blood Agar Base in Roux flasks. After 24 hr at 37 C, the growth was harvested in a minimal amount of sterile distilled water and frozen in glass vials containing 2 ml each. For aerosolization, the stock culture was regrown on Blood Agar Base, harvested, and diluted to 10⁶ organisms per milliliter in sterile water.

The aerosol chamber was a 200-liter plastic container which was inserted into a microbiological safety hood. A modified University of Chicago Toxicity Laboratory atomizer was used to produce the aerosol. The liquid culture was fed from a 50-ml syringe, the plunger of which was activated by a revolving threaded rod propelled by a 1-rev/min synchronous electric motor. The atomizer delivered 0.4 ml of culture mixed in 32.5 liters per min of air into the chamber. The chamber air was maintained at 73 ± 2 C and 80 ± 5% relative humidity (RH).

Animals were exposed for 10 min to the bacterial aerosol, in particle size of 1 to 5 μ. After the exposure, aerosol production was stopped, and the animals were air-washed for 15 min.

The source of nitrogen dioxide was a gas cylinder containing 10,000 ppm of nitrogen dioxide in air. The flow of the gas was measured on passage from the cylinder to a mixing chamber where it was further diluted with filtered air. For acute exposures, the nitrogen dioxide-air mixture was introduced into a 3.5-ft³ glass aquarium. For chronic exposure, a walk-in type chamber was used.

Two basic experimental procedures were employed with the use of mice in groups of 10 and hamsters in groups of 6. To determine the effect of pre-exposure to nitrogen dioxide on resistance, experimental animals were exposed to the gas for a 2-hr period before the challenge with the infectious aerosol. To study the effect of nitrogen dioxide on the course of the infection, animals challenged with *K. pneumoniae* were exposed for 2 hr to the gas. The animals were observed for 14

TABLE 1. *Mortality of Swiss albino mice exposed for 2 hr to nitrogen dioxide 1 hr before infectious challenge*

NO₂	Mortality: deaths/total		Change	P
	Infected controls	Exptl groups		
ppm			%	
1.5	71/150	86/150	21.1	
2.5	135/400	158/400	16.8	
3.5	44/100	98/100	122.7	<0.05
5	112/250	234/250	108.9	<0.05
10	19/40	39/40	105.3	<0.05
15	13/40	35/40	169.2	<0.05
25	40/100	46/50	130.0	<0.05

days after aerosol challenge, during which time mortality and survival time data were recorded. Autopsies were performed on all animals at the time of death, and randomly selected lung tissues were subjected to histopathological examination. Blood-agar plates were streaked with heart blood to confirm *K. pneumoniae* as the cause of death. Animals surviving the 14-day observation period were sacrificed and examined in the same way.

In all experiments, control groups of animals were exposed either to nitrogen dioxide or to the infectious agent, simultaneously with the experimental animals. Accordingly, results could be compared on the basis of individual test exposures or could be pooled for statistical analysis. The mortality and the survival data were analyzed statistically by the *t* test. Significance is reported at *P* <0.05. There were no deaths in any of the animals exposed to nitrogen dioxide only. The mortality in the animals challenged with *K. pneumoniae* was only approximately 40%.

Swiss Albino Mice

The effect of a 2-hr exposure to nitrogen dioxide on resistance of Swiss albino mice to infection has been reported in detail in previous publications (8, 9) and will be discussed here only briefly. Table 1 summarizes the data on the effect of nitrogen dioxide in concentrations ranging from 1.5 to 25 ppm. The time interval between the termination of the nitrogen dioxide exposure and the infectious challenge was 1 hr or less. Mice not exposed to the gas but challenged with *K. pneumoniae* aerosol simultaneously with the experimental mice served as controls.

Based on the results shown in Table 1, a threshold value was determined at which the exposure to nitrogen dioxide reduces the resistance of Swiss albino mice to respiratory infection. The threshold is approximately 3 ppm.
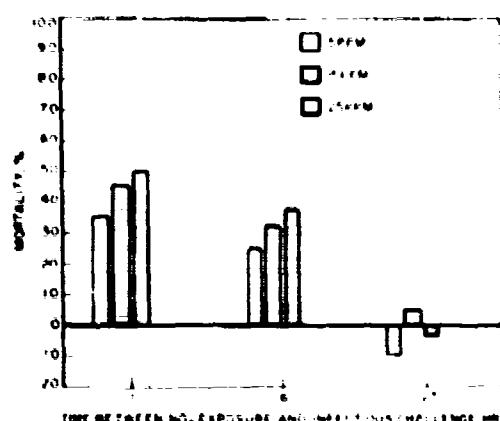
Fig. 1. *Mortality rates corrected for control mortality in mice exposed for 2 hr to various concentrations of NO₂ prior to challenge with infectious agent*

TABLE 2. *Mortality of Swiss albino mice exposed for 2 hr to nitrogen dioxide 1 to 24 hr after infectious challenge*

NO₂	Time between challenge and exposure	Mortality		Survival time
		Deaths/total	Per cent	
ppm	hr			days
0	0	27/60	45.0	10.9
2.5	1	30/40	90.0	9
2.5	6	33/60	55.0	9.8
2.5	24	32/60	53.3	10.2
0	0	15/30	50.0	10.6
25	1	30/30	100.0*	4.7*
25	6	28/30	93.3*	6.0*
25	24	26/30	86.6*	7.6*

* Significant at P < 0.05

The acute exposure appeared to produce an all-or-none response. There was no effect at 2.5 ppm, a complete effect at 3.5 ppm, and a minimal effect at higher concentrations. The mean survival time of the infected controls, calculated on the basis of a maximal 14-day survival, was 11.1 days. The survival time was not affected by exposure to nitrogen dioxide concentrations of up to 2.5 ppm, but it was reduced to 5.5 days at concentrations ranging from 3.5 to 25 ppm.

No deaths occurred in the mice exposed to nitrogen dioxide only, irrespective of the concentrations used. In mice exposed to 5 ppm or more, the lungs were congested to various degrees, and the veins and capillaries of the lungs were dilated. Concentrations of less than 5 ppm produced little, if any, damage.

For the histopathological examinations, the mice were sacrificed within 1 hr after the termination of exposure to nitrogen dioxide. On the few occasions when the sacrifice was delayed for 24 or 48 hr, pathological findings were reduced or absent. Thus, it was of interest to determine whether the effect of acute exposure to nitrogen dioxide on resistance to infection is transitory. To study this parameter, the time interval between the termination of the exposure to nitrogen dioxide and the infectious challenge was extended from 1 hr to 6 and 27 hr. The data in Fig. 1 show that the decrease in resistance was not permanent and disappeared within 27 hr after the termination of the nitrogen dioxide exposure. The persistence of the effect was not influenced by the concentrations of gas used within the 5 to 25 ppm range. It can be assumed, therefore, that nitrogen dioxide produces a temporary damage to

the defense mechanisms; this damage disappears within 24 hr.

Concentrations of 2.5 or 25 ppm were used to study the effect of a 2-hr exposure to nitrogen dioxide on mortality of previously infected mice. The mortality increased from 50%, in the control mice to 100%; in the mice exposed to 25 ppm of nitrogen dioxide. At 2.5 ppm, there was no significant difference in mortality between control and exposed mice. Delaying the exposure to nitrogen dioxide for 6 or 24 hr after the infectious challenge did not significantly alter the mortality increase in mice exposed to 25 ppm (Table 2).

Inbred Mice

Increased immunity due to genetically conditioned natural resistance may manifest itself as resistance to invasion by bacteria or as increased ability to produce bacterial antibodies. In studies of acute toxicity of oxides of nitrogen, Gray and co-workers (13) observed appreciable variations in the response of rats obtained from different sources. An exposure difference of 40 ppm was required to produce an LD₅₀ in groups of rats from two different sources. In studies of chronic exposures, Wagner et al. (28) found no effect that could be attributed to the nitrogen dioxide exposures, and therefore strain difference among HLA, C₃BL/6, and CAF₁/Jax mice were not observed.

The effect of a 2-hr exposure to 5 ppm of nitrogen dioxide on resistance to respiratory infection was determined in BDF₁, BALB/c, C₃BL/c, and LAF₁ mice. Groups of mice from each inbred strain were exposed simultaneously with Swiss albino Webster strain mice to nitrogen dioxide, and either before or after this exposure were challenged with airborne *K. pneumoniae*.

TABLE 3 Mortality of various mouse strains exposed to nitrogen dioxide and infectious challenge

	NO₂ before challenge			NO₂ after challenge		
Mouse strain	Mortality (death/total)			Mortality (death/total)		
	Infected controls	Exptl group	Change	Infected controls	Exptl group	Change
SA	164/390	262/390	59.6	247/630	372/630	50.8
BDF₁	31/120	40/120	29.1	66/240	105/240	58.9
BALB/c	48/100	72/100	50.0	43/100	62/100	44.2
C₃BL/c	24/70	36/70	49.9	29/130	34/130	17.4
LAF₁	40/100	50/100	25.0	52/160	91/160	74.8

' Significant (P < 0.05) change due to NO₂ exposure
' Significant (P < 0.05) change from Swiss albino mice

The interval between these two treatments was 1 hr or less.

Table 3 summarizes the results obtained on the basis of a minimum of eight replicate exposures. The data can be considered from two standpoints. One relates to strain differences in resistance to the infection per se; the other, to effects of exposure to nitrogen dioxide on resistance.

BDF₁ and C₃BL/c mice were more resistant to the infection than the other two strains, as measured by mortality. The mean survival times of the two more resistant strains were 12.2 and 12.0 days, respectively. Compared with the 10.9 mean survival time of the Swiss albino mice, this increase was statistically significant (P < 0.05). The other two inbred strains showed mortalities and mean survival times similar to those of the Swiss albino mice.

Exposure to nitrogen dioxide followed by infectious challenge significantly increased mortality in the Swiss albino, BALB/c, and C₃BL/c mice. The increases due to the exposure were 59.6, 50.0, and 49.9%, respectively. The mortality of BDF₁ and LAF₁ mice also increased to 29.1 and 25.0%, respectively, but the differences were not significant. The LAF₁ data, however, must be considered with caution. In this group of experiments, only a small increase in mortality was observed upon exposure to nitrogen dioxide of the Swiss albino mice challenged at the same time as the LAF₁ mice.

Exposure to nitrogen dioxide prior to infectious challenge increased mortality in all five strains. The increase was significant in all but the C₃BL/c strain.

The data suggest that mouse strain differences are of importance in resistance to infection produced by K. pneumoniae. The damage produced by nitrogen dioxide, on the other hand, is not

TABLE 4 Mortality of hamsters exposed for 2 hr to nitrogen dioxide 1 hr before infectious challenge

	Mortality (deaths/total)			
NO₂	Infected controls	Exptl group	Change	P
ppm				
5	18/96	22/96	21.8	
15	11/126	19/126	73.5	
25	15/72	10/72	-33.2	
35	9/90	40/90	389.0	<0.001
40	6/72	28/72	368.7	<0.005
50	11/138	44/138	298.7	<0.001
65	12/96	61/96	408.0	<0.001

closely related to strain differences. In all instances, mice exposed to nitrogen dioxide either before or after the infectious challenge showed increased mortality. The 5 ppm of nitrogen dioxide did not produce any significant damage to the respiratory system, as determined by histopathological examination of the lungs.

Hamsters

Golden hamsters have a high natural resistance to K. pneumoniae infection initiated by the respiratory route. Inhaled respiratory doses as high as 30,000 organisms produced only 12%, mortality in our studies: of 690 hamsters challenged with the infectious agent, 82 died. The same challenge dose repeatedly produced 100% mortality in Swiss albino mice used as controls.

A 2-hr exposure to high levels of nitrogen dioxide terminated 1 hr prior to infectious challenge significantly altered the resistance of hamsters. As shown in Table 4, concentrations ranging from 5 to 25 ppm caused some increase in mortality, but it was not significant. Concentra

tions ranging from 35 to 65 ppm increased mortality significantly; the mortality of the control group was 9.6%, but this increased to 44.7% in the exposed group.

Exposure to nitrogen dioxide apparently is a significant factor in hamsters' resistance to respiratory infection by *K. pneumoniae*. The 10-fold increase in nitrogen dioxide required to produce this effect in hamsters, as compared with mice, cannot be explained at present. It can be related only pertially to the differences in body weights and respiratory volumes of these two species. However, the all-or-none response and the absence of a graded dose response are similar in the two species.

Squirrel Monkeys

Increased mortality was observed in preliminary studies with squirrel monkeys exposed for 2 hr to approximately 40 ppm of nitrogen dioxide followed by respiratory challenge. Three groups of monkeys were included in the experiments: one challenged with airborne *K. pneumoniae* only, one exposed to nitrogen dioxide only, and one exposed to nitrogen dioxide and within 1 hr challenged with the infectious agent. Deaths occurred only in the last group; of the five monkeys exposed to both stresses, three died.

EFFECT OF RETENTION OF BACTERIA IN LUNGS

The response of the respiratory system to infectious agents involves the activation of such gross defense mechanisms as cough, alterations in the respiratory functions, phagocytosis, mucus flow, and alterations in ciliary activity.

Under normal conditions, inhaled bacteria are
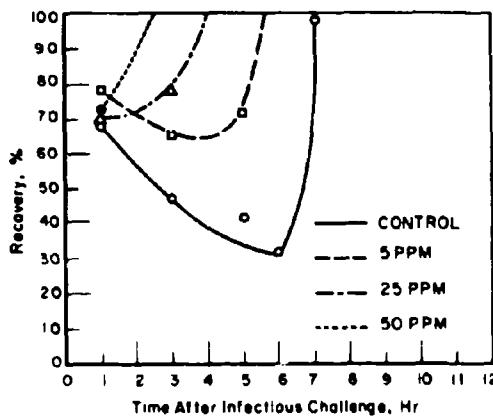
FIG. 2. Recovery of Klebsiella pneumoniae from lungs of mice exposed to NO₂

deposited upon mucus, which, through ciliary action, is constantly moved from the deeper part of the lung toward the larynx. Thus, ciliary movement combined with mucus secretion normally prevents an accumulation of particles in the tracheobronchial tree.

This defense mechanism against invasion by bacteria can be altered. Drying, for example, markedly impairs the mobility and the effectiveness of ciliary actions. Irritant gases, such as ozone, sulfur dioxide, ammonia, and nitrogen dioxide, have been reported to interfere with ciliary movement (2, 6, 15). Thus, one parameter that can be utilized to determine the toxicity or the effect of irritant gases is their action on the ciliated epithelium of the respiratory tract.

The role of phagocytosis as a clearance mechanism of inhaled dust particles is well recognized. Defense against bacterial infection in the lung is similar to defense against dusts. In both cases, alveolar macrophages play a key role in the clearance (16). Green and Kass (11) impaired pulmonary clearance mechanisms in mice by a variety of stresses: hypoxia, cold, corticosteroid injection, and ethyl alcohol intoxication. The inhibition of clearance depended on the type and the extent of the treatment and on the bacterial species being cleared.

To study the effect of nitrogen dioxide on clearance of bacteria by the lower respiratory tract, *K. pneumoniae* was used as the infectious agent. Swiss albino mice and hamsters were exposed for 2 hr to nitrogen dioxide in concentrations ranging from 5 to 50 ppm. Within 1 hr after the exposure, they were challenged with the infectious aerosol. Groups of animals were sacrificed immediately after the infectious challenge. The lungs were removed aseptically from each animal, homogenized in sterile saline, and cultured quantitatively. The initial counts were assumed to be 100% recovery. Control animals as well as animals exposed to the nitrogen dioxide were sacrificed at 1, 3, 5, 6, 7, and 8 hr after the combined treatment. The mean number of bacteria present in the lungs of each group of animals was plotted against the time elapsed after the infectious challenge.

Figure 2 shows the data obtained in mice. Recoveries of *K. pneumoniae* from the lungs of mice exceeding 100% are not shown in the figures. However, they were used in construction of the recovery curves. The mean recovery of bacteria from the lungs of control mice challenged only with the infectious aerosol showed a similar pattern in three replicate tests. The bacterial population was markedly reduced (a range of 65 to 90% was observed) during the first 5 to 6 hr after
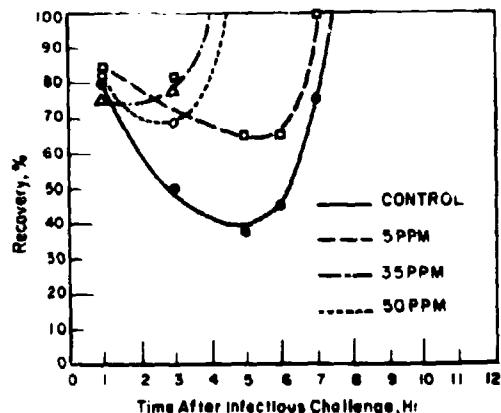
FIG. 3 Recovery of Klebsiella pneumoniae from lungs of hamsters exposed to NO₂.

challenge. Thereafter, the population increased and reached the initial concentration after 6 to 8 hr.

In mice exposed to 5 ppm of nitrogen dioxide, the 100% concentration was reached within 5.5 hr. In mice exposed to 25 ppm, the bacterial population decreased during the 1st hr and increased thereafter; the 100% concentration was reached within 3.3 hr. In mice exposed to 50 ppm, the 100% concentration was reached in 2.3 hr. A 4- to 6-log increase in concentration of *K. pneumoniae* occurred in mice 24 hr after the infectious challenge, irrespective of the previous treatment.

Figure 3 shows that similar results were obtained in hamsters. In control hamsters not exposed to nitrogen dioxide, gradual reduction of bacteria occurred during the first 5 hr, and the initial concentration point was reached after 7.3 hr. In hamsters exposed to 5 ppm, this 100% point was observed after 6.4 hr, and in those exposed to 35 ppm, after 2.9 hr.

In the experiments, mice or hamsters exposed to nitrogen dioxide 1 hr before infection were challenged with the infectious aerosol simultaneously with control animals not exposed to the gas. Both groups of animals were thus exposed to the same quantity of *K. pneumoniae*. However, as shown in Table 5, the initial recoveries of *K. pneumoniae* from lungs varied widely. In all instances, fewer organisms were recovered from the animals exposed to nitrogen dioxide. In hamsters, the decrease in organisms appears to be related to the concentration of nitrogen dioxide. At 5 ppm, the recovery was 75% of that in the controls; at 35 ppm, 58%; and at 50 ppm, 44%. However, experiments were not conducted to determine the statistical significance of this relation. In mice, the recovery was approximately

71% of that in controls, irrespective of the nitrogen dioxide concentration.

While the increased mortality in animals exposed to nitrogen dioxide can in part be explained by damage to the ciliary activity and the phagocytic activity, the lower recovery of inhaled bacteria from the lungs of animals exposed to nitrogen dioxide cannot be ascribed to this type of damage. Also, because of the absence of appreciable pulmonary edema in animals exposed to nitrogen dioxide, a dilution effect can be discounted. Three theoretical explanations are possible. One is that the nitrogen dioxide remaining in the lungs inactivates the bacteria in situ. This supposition is questionable, because larger amounts of nitrogen dioxide are usually required to produce any effects on bacteria. Another explanation is that a protective film forms in the respiratory system as a result of inhalation of the gas; this film would make the recovery of bacteria from the lung tissue more difficult. The third possibility is that the respiratory functions are modified by inhalation of nitrogen dioxide.

Our studies in squirrel monkeys showed that exposure to nitrogen dioxide increased the respiratory rate and decreased the tidal volume. Although the animal was breathing more frequently, the breathing was shallow. Thus, it is possible that the bacteria do not penetrate into the alveoli in the same quantities as in normal animals.

The tidal volume of a monkey exposed for 2 hr to nitrogen dioxide is shown in Fig. 4. The monkey was placed in a restraining chair, a mask was fastened to its face, and it was exposed to filtered air for 30 min. Without any interruption, 35 ppm of nitrogen dioxide was introduced into the air, and the respiratory functions were measured with a spirometer and a dual-channel recorder. After the exposure, the tidal volume was approximately 72% of the initial value.

EFFECT ON LACTIC DEHYDROGENASE (LDH) ISOENZYMES

The LDH enzyme system plays a principal role in the glycolytic cycle for the conversion of stored

TABLE 5. *Retention of Klebsiella pneumoniae in lungs of mice and hamsters*

NO₂	No. of organisms/g of lung tissue			
	Control hamsters	Exptl hamsters	Control mice	Exptl mice
ppm				
5	3,948	2,966	3,366	2,457
25	—	—	1,530	1,092
35	7,284	4,253	—	—
50	7,142	3,122	1,289	883
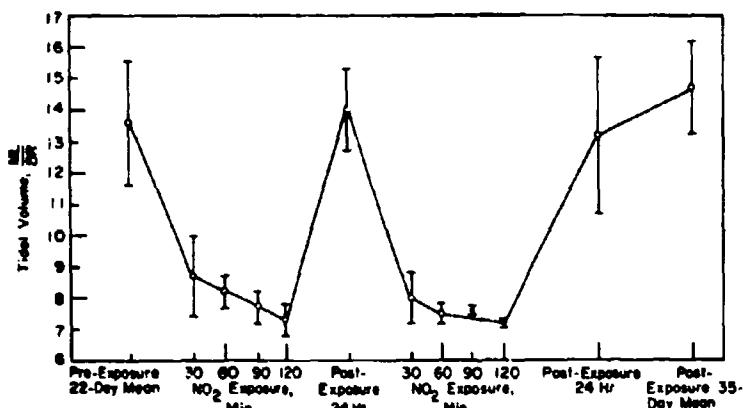
FIG. 4. *Mean tidal volumes of monkey exposed to 35 ppm of NO_2.*

energy. Recently, the enzyme has been separated electrophoretically into five components defined as LDH isoenzymes (29). Diseases, such as cardiac infarction (30), hepatitis (25), and cancer (23), produce abnormal serum and tissue isoenzyme patterns that are indicative of the tissues affected.

Exploratory studies were conducted to determine whether exposure to nitrogen dioxide and infection with *K. pneumoniae* produce an atypical LDH isoenzyme pattern in serum or selected tissues and whether the pattern is indicative of the resulting pathology. The limited number of animals (four) used per point did not permit an exhaustive analysis of the data. However, the differences obtained are large enough to suggest trends and to form the base for additional experimentation. Hamsters were exposed to 5 and 35 ppm of nitrogen dioxide for 2 hr and examined frequently over a 72-hr period.

Heart tissue was removed from each hamster; the LDH enzyme was extracted and resolved into isoenzyme components. Approximately 1 hr after exposure, in either group the enzymatic activity of isoenzymes 1 and 2 was reduced and remained depressed for approximately 1 day. Correspondingly, the isoenzyme activity in bands 4 and 5 increased. This period of altered isoenzyme activity coincides with the period of maximal susceptibility to respiratory infection after exposure to nitrogen dioxide. Hamsters subjected to infectious challenge only did not experience these alterations.

The livers were removed and the LDH isoenzyme was extracted. Although there was a large variability among the hamsters in a group, the overall trend was clear. Hamsters exposed to 5 or 35 ppm sh· ·d a decrease of approximately 50'; in band 1 and 2 isoenzyme activity 5 hr after exposure.

Exploratory studies were also conducted with squirrel monkeys. One monkey was exposed to 35 ppm for 2 hr and subsequently was infected intravenously with *K. pneumoniae*. The LDH activity in the lung tissue increased 5-fold 24 hr after exposure. The increase in activity was accompanied by a marked and disproportionate increase in the LDH activity in bands 4 and 5.

Two monkeys exposed to 50 ppm of nitrogen dioxide and to *K. pneumoniae* aerosol displayed similar isoenzyme alterations. Upon autopsy, the lung tissue appeared gray, with patches of marked reddish congestion clearly demarcated from the gray areas. Tissue sections from both gray and red areas showed much interstitial and intralveolar edema, with congestion and cellular infiltration. Tissue excised from each of these areas produced abnormal isoenzyme patterns, each very different from the other. The red area produced one major isoenzyme band, band 5, with considerably reduced activity in the remaining isoenzyme fractions.

Current theories suggest that the LDH molecule is a tetrameric peptide molecule and that the synthesis of the molecule is controlled by two genes (5). One gene is responsible for the synthesis of LDH isoenzyme 1 and one for the synthesis of LDH isoenzyme 5. The three remaining isoenzymes are merely combinations of isoenzymes 1 and 5. Cahn, Kaplan, and associates (5) suggest that LDH isoenzyme 1 is associated with cells undergoing aerobic metabolism and LDH isoenzyme 5 with cells functioning anaerobically. Brody and Engel (4) have demonstrated that LDH activity is associated with the mitochondrial membrane and is readily dissociated when the tissue is manipulated during fixation. Therefore, rupture of the cellular membrane (cell death) would be indicated by an increase in serum LDH activity.

Altered metabolism induced by stress may be related to increased enzyme activity and altered isoenzyme ratios. Recently, Vesell (27) has published contradictory results. Studies conducted with nucleated red blood cells and normal red blood cells devoid of a nucleus indicated that LDH isoenzyme band 5 is located in the nucleus, and isoenzymes 1, 2, and 3 are located in the cytoplasm. Vesell therefore takes exception to the theories regarding the relationship of aerobic-anaerobic metabolism to isoenzymes 1 and 5.

The preliminary data acquired to date do not permit interpretation of the cellular mechanisms involved. Nevertheless, it is apparent that alteration of the isoenzyme ratios is related to pathology, and may ultimately provide information regarding altered cellular metabolism induced by the nitrogen dioxide and infectious challenge stress.

EFFECT OF CHRONIC EXPOSURE

Exposure to low levels of pollutants over extended periods of time are a threat to heavily populated communities. Air pollution surveys indicate a maximal concentration of 3.5 ppm of nitrogen dioxide. Daily variations in the concentration of nitrogen dioxide in a polluted atmosphere result from varying emission rates, wind velocity and direction, height of inversion layer, etc. The average 8-hr levels of oxides of nitrogen in one urban area on days with significant air pollution ranged from 0.1 to 0.5 ppm (26).

Several investigators have reported on the effect of chronic and intermittent exposures to nitrogen dioxide. Ronzani (24) concluded that repeated daily exposures to 100 ppm had no distinct acute effect in animals. Gray et al. (12) exposed rats to 9 to 14 ppm for 4 hr per day, 5 days per week, for 6 weeks. They observed an inflammatory condition spread throughout the entire respiratory tract. The same authors (13) found no evidence of pathology in rats, guinea pigs, and mice exposed daily for 6 months to 4 ppm.

Wagner et al. (28) exposed dogs, guinea pigs, rabbits, rats, hamsters, and mice to 1, 5, and 25 ppm for periods up to 18 months. At no exposure level did changes in body weight, hematological value, or biochemical index vary significantly from the control data. The respiratory functions in exposed rabbits were equivalent to those in the controls, with the exception of the 25-ppm group, which indicated a slight and transitory elevation in mean oxygen consumption. Detailed histological examination of tissues of animals sacrificed at various time intervals presented no evidence that nitrogen dioxide had any morphological effect. Their studies with a strain of mice

TABLE 6. *Effect of continuous exposure to 0.5 ppm of nitrogen dioxide on mortality of mice challenged with Klebsiella pneumoniae*

NO₂ exposure	Mortality (deaths/total)			P
	Infected controls	Exptl group	Change	
			%	
7 days	187/280	189/280	1.0	
14 days	81/180	92/180	13.6	
1 month	26/60	34/60	30.9	
2 months	68/100	78/100	14.7	
3 months	64/100	92/100	43.7	<0.05
6 months	24/50	44/50	83.3	<0.001
9 months	38/70	49/70	28.9	<0.001

susceptible to spontaneous pulmonary tumor suggested a possible tumorigenic accelerating capacity of nitrogen dioxide.

In our studies, Swiss albino mice were exposed continuously, 24 hr per day, to 0.5 ppm of nitrogen dioxide. Three times a week, the mice were removed from the chamber for approximately 1 hr for maintenance and feeding. After various periods of nitrogen dioxide exposure, the mice were challenged with the aerosol of *K. pneumoniae* and maintained in a clean air atmosphere for 14 days after the challenge. Control animals were of the same age as the experimental mice, and were treated identically with the exception of the nitrogen dioxide exposure. The data in Table 6 show an increase in susceptibility to infection after 3 months of exposure to the gas. Some degree of linearity was observed when the arc transformed differences in mortalities were plotted against the duration of exposure to nitrogen dioxide (Fig. 5).

The effect of continuous and intermittent exposure to 0.5 ppm of nitrogen dioxide over a 30-day period was investigated. After 30 days of continuous exposure, Swiss albino mice were challenged by the respiratory route with airborne *K. pneumoniae*. The deaths were recorded during the next 14-day holding period at ambient atmosphere. For intermittent exposure, mice were exposed to 0.5 ppm for 6 hr a day, 5 days a week, for a total of 30 days before the infectious challenge. The data summarized in Table 7 show the significant mortality increase due to the intermittent exposure.

Exposure of Swiss albino mice to 1.5 ppm of nitrogen dioxide for periods ranging from 2 hr to 3 months prior to the infectious challenge resulted in mortality shown in Table 8. The increase in mortality was significant after exposures of 8 hr or longer. A corresponding reduction in
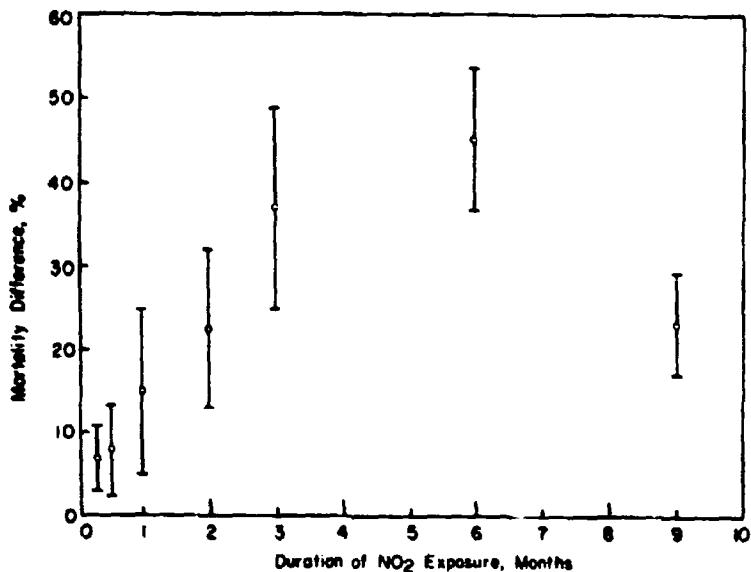
FIG. 5. *Mortality difference versus chronic exposure to NO_2.*

TABLE 7. *Effect of continuous and intermittent exposure to 0.5 ppm of nitrogen dioxide for 30 days on mortality of infected mice*

| NO₂ exposure | Mortality | | Change | Survival time |
	Deaths/total	Per cent		
			%	days
Continuous				
Controls	32/80	40.0		10.8
Experimental				
group	79/140	56.4	41.0	10.2
Intermittent				
Controls	22/60	36.6		12.2
Experimental				
group	57/80	71.3	94.8ᵃ	9.2

ᵃ Significant at $P < 0.05$.

TABLE 8. *Effect of continuous exposure to 1.5 ppm of nitrogen dioxide on mortality of mice challenged with Klebsiella pneumoniae*

| NO₂ exposure | Mortality (deaths/total) | | Change |
	Infected controls	Expt'l group	
			%
2 hr	45/90	51/90	13.4
8 hr	45/90	67/90	48.8ᵃ
24 hr	45/90	59/90	31.2ᵃ
7 days	20/40	28/40	40.0ᵃ
14 days	17/40	39/40	129.4ᵃ
90 days	23/90	70/100	52.3ᵃ

ᵃ Significant at $P < 0.05$.

the survival time occurred in all groups except the one exposed to nitrogen dioxide for 2 hr.

Exposure of infected mice to 1.5 ppm of nitrogen dioxide after the infectious challenge also increased mortality. The mortality in the control group challenged with the infectious agent only was 45%. Aft· xposure to nitrogen dioxide for 2, 8, or 24 hr, the mortality rates were 80.0, 88.3 and 73.3%, respectively. The respective increases in mortality were 77.8, 96.2, and 62.9%, all three values being significant.

The significance of pre-exposure to nitrogen dioxide is further illustrated in Fig. 6. Three groups of mice were used. The one serving as the

control was challenged with *K. pneumoniae* aerosol and maintained in clean air after the infection. The second was infected and placed immediately after the challenge in an atmosphere of 1.5 ppm of nitrogen dioxide. The third was exposed to nitrogen dioxide for 24 hr, challenged, and returned to the 1.5-ppm atmosphere. Although the mortality of both groups exposed to nitrogen dioxide was higher than that of the control group, mice exposed to the gas both before and after the infectious challenge died faster, and ultimately the mortality in this group was the highest. The mortality at the end of the 30-day holding period was 58.3% for controls, 78.3% for mice exposed to nitrogen dioxide after the in-
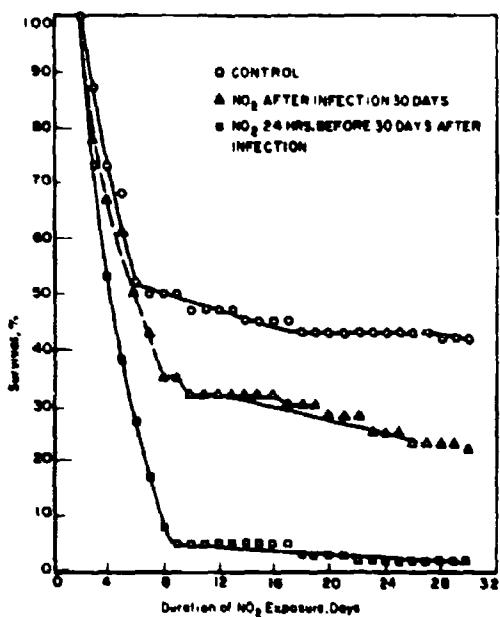
Fig. 6. *Effect of pre-exposure of mice to nitrogen dioxide on resistance to Klebsiella pneumoniae.*

fectious challenge, and 98.3% for mice exposed to nitrogen dioxide both before and after the infectious challenge.

Effect of Age

The effect of age on resistance to *K. pneumoniae* infection was investigated. Young mice 6 to 8 weeks old and weighing 20 ± 2 g, mice 6 months old and weighing 32 ± 2 g, and mice 9 months old and weighing 39 ± 2 g were maintained at ambient atmosphere and were challenged simultaneously with the infectious agent. Deaths were recorded for 14 days after the challenge. No significant differences were observed in the mortality rates among these groups. The mortality of the 6-month-old mice was 48.0; the 9-month-old mice, 54.2%; and the 6 to 8-week-old mice, 52.5%.

Discussion and Conclusions

The effects of exposure to nitrogen dioxide on man and on animals are confined almost exclusively to the respiratory tract. With increasing dosage, the progressive effects of this gas are: odor perception, nasal irritation, difficulty in breathing, acute respiratory irritation, edema, and death. Experimental and epidemiological data pertaining to nitrogen dioxide effects in man are sparse, especially in the low concentration level found in community air pollution.

In most species of laboratory animals, concentrations of nitrogen dioxide above 200 ppm produce death even after a single 5- to 15-min exposure. Continuous 30- to 60-min exposures to 100 to 200 ppm or 8-hr exposures to 50 ppm also produce death. Intermittent exposures of less than 50 ppm, on the other hand, are not fatal. Thus, it appears that the existence of a recovery period reduces mortality.

Lower concentrations, 10 to 20 ppm, produce pathological changes in the lungs. Continuous exposures to 5 or 10 ppm result in changes in the bronchial epithelium; lower concentrations produce only minor changes. Freeman and Haydon (10) observed minor changes in the bronchial epithelium after continuous exposure to 4 ppm for 20 weeks. Balchum et al. (1) showed that exposure of guinea pigs to 5 ppm produced minor pulmonary changes and demonstrated the development of circulating substances capable of agglutinating normal lung proteins.

The work reported in this paper suggests a more sensitive indicator of biological effects of nitrogen dioxide, namely, a synergistic effect or secondary effect, demonstrated by reduction in resistance to infection. A single 2-hr exposure of Swiss albino Webster strain mice or of inbred mice to 3.5 ppm of nitrogen dioxide before or after respiratory challenge with aerosol of *K. pneumoniae* significantly increased mortality. To produce the same effect in hamsters and squirrel monkeys, 35 ppm was required during the 2-hr exposure period. The effect of the single 2-hr exposure was not persistent, and a return to normal resistance to the infection was observed within 24 hr after the exposure to nitrogen dioxide.

Continuous exposures to 0.5 ppm for 3 months or longer as well as intermittent daily exposures over a 30-day period produced the same effect in mice.

Exploratory studies conducted to define the mechanisms responsible for the increased susceptibility to infection suggest that exposure to nitrogen dioxide permits better colonization of bacteria in the lungs of mice and hamsters.

Exposure to 25 to 3ᶜ ppm of nitrogen dioxide affected the pulmonary function in squirrel monkeys. Similar observations in guinea pigs were reported by Murphy et al. (19). The respiratory rate increased and the tidal volume decreased in guinea pigs exposed to 5.2 and 13.0 ppm of nitrogen dioxide. The time of onset of the respiratory changes was inversely related to the concentration of the inhaled gas. When the guinea pigs were returned to clean air, the pulmonary function gradually returned to the pre-exposure level.

Extrapolation of the effects of nitrogen dioxide on resistance to *K. pneumoniae* infection of man, or that due to other species of pathogenic miro-organisms, can be speculative only. However, the work is significant in pointing to possible relationships between air pollutants and changes in resistance to respiratory infection.

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Attenuation of Aerosolized Yellow Fever Virus After Passage in Cell Culture

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INTRODUCTION

Reports in the literature, recently reviewed by Musgay (8), contain numerous examples of alterations in the properties of arboviruses as a result of their passage in various host systems. Countless other observations of this type undoubtedly have been made but not reported. Early accounts of alterations in virulence of yellow fever virus (YFV) as a result of propagation in vitro have been described by Lloyd, Theiler, and Ricci (6) and by Theiler and Smith (11). Studies by Theiler and his associates led to the isolation and eventual use of the well-known 17-D strain for human vaccination. A more detailed description of the development of various attenuated strains of YFV may be found in a review by Theiler (10). More recently, Hallauer (1) reported losses in virulence of the 17-D strain for mice and of the Asibi strain for monkeys after passage in KB cell cultures. Following this, Schindler and Hallauer (9) described additional losses in the viscerotropism of Asibi strain variants obtained after prolonged passage in KB cells. These authors also reported the isolation from human cell lines of a 17-D viral substrain whose virulence for monkeys was reduced to a degree that has apparently not been found in any other 17-D strains. Hardy (2) demonstrated attenuation of the Asibi strain for monkeys with viral isolates obtained after serial passage in HeLa cells. In keeping with the general subject of this symposium, we should like to present some observations on changes in the properties of yellow fever virus that were discernible upon aerosolization of preparations after growth in HeLa cells.

A major consideration in determining our experimental approach was the prevailing lack of information on the behavior of airborne virus that previously had undergone routine passages in cell culture in the laboratory. Information of this kind has broad applications not only for the viral geneticist but for those who are engaged in the problems of laboratory safety. To carry out a meaningful study, it was recognized that a comparison of properties of virus when in suspension with those of virus that was aerosolized was needed. The results will show that the dominant characteristic of virus that was serially passed in cell culture was its decline in virulence. Other properties that were also found to change appeared to vary in parallel with the loss in virulence. It seems probable that some of these alterations may have resulted in, or at least contributed to, the decline in capability of the virus to induce lethal infections.

EXPERIMENTAL APPROACH

Two years ago at Fort Detrick, Hardy (2) demonstrated that his strain of yellow fever virus, which was ordinarily noncytopathic in cell culture and lethal for monkeys, induced cell lysis and became attenuated for these animals after six serial passages in HeLa cells. Subsequently, results reported by Miller et al. (7) indicated the feasibility of performing studies on aerosolized yellow fever virus after it had proliferated in HeLa

TABLE 1. Properties of typical and atypical yellow fever virus populations obtained after one and three serial passages in HeLa cells

Population	Titer in culture, MICLD50 (logs)	Day of maximal titer	Cell lysis in culture	Per cent recovery in aerosol		Monkey lethality	
				40-50% RH[a]	70-80% RH	40-50% RH	70-80% RH
Ty-1[b]	7.1	5	Negative	43.2	46.7	Lethal at 10 MICLD50	
ATy-1[c]	8.1	5	Positive	9.7	28.2	Lethal at 10 MICLD50	
Ty-3[d]	8.8	3	Positive	9.8	31.5		Nonlethal at >52.5 MICLD50
Aty-3[e]	6.9	3	Negative	19.7	12.3		Nonlethal at 800 MICLD50

[a] Relative humidity.
[b] "Typical" viral population, representative of harvests obtained in 11 of 12 experiments.
[c] "Atypical" viral population, representative of a harvest obtained in 1 of 12 experiments.
[d] "Typical" viral population, representative of harvests obtained in four of six experiments.
[e] "Atypical" viral population, representative of harvests obtained in two of six experiments.

cells. Encouraged by the results of these efforts, we designed experiments to characterize properties of yellow fever virus in greater detail during serial passage in cell culture. An effort was made during this work to study properties of virus in both the pre- and postaerosolized state and to determine whether any correlation existed among the properties that were altered as a result of passage in vitro. It was found that the viral changes that were reported by Hardy, as well as additional previously undescribed changes, actually began to occur as early as the third serial passage in HeLa cells. We observed alterations in the ability of the virus to grow and induce cell lysis in HeLa cell monolayers, confirming Hardy's published work. We also found changes in the stability of infected cell culture preparations upon aerosolization, and in the level of virulence of such products for rhesus monkeys.

ALTERATIONS IN VIRAL PROPERTIES AFTER CULTIVATION IN HeLa CELLS

Recovery of "Typical" and "Atypical" First- and Third-Passage Viral Populations

During the course of our first series of studies, viral products that were prepared in the same or similar manner during either one or three passages did not repeatedly demonstrate the same properties. In other words, although the majority of viral populations behaved in a typical fashion, infrequent atypical populations could be recovered. These proved to be fortuitous events, because this not only allowed for an assessment of the differences between typical one- and three-passage

harvests, but, by studying the incidence of variation among properties of typical and atypical viral populations prepared at the same passage level, we were able to gain some insight into possible relationships that existed among individual genetic markers. Of considerable interest was the indication that certain of the properties that were encountered in vitro could be correlated with properties displayed by the virus in aerosols. Examples are shown in Table 1.

It can be seen that typical first-passage preparations possessed titers of approximately 10[7] MICLD50 that were obtained on day 5 in the absence of cell lysis. This preparation showed maximal stability during aerosolization at 50% relative humidity (RH) and was lethal for monkeys by the respiratory route. In contrast, typical third-passage preparations, represented in the third column, possessed titers of 10[9] MICLD50 (or greater) that were obtained on day 3 attended by cell lysis. The viral recoveries obtained at 50% RH were significantly lower ($P < 5\%$) than those obtained at 80% RH. These preparations were not lethal for monkeys by the respiratory route. In column two, properties of an atypical first-passage preparation are shown. A 1-log increase in titer to 10[8] MICLD50 was obtained at day 5, accompanied by cell lysis. This preparation was significantly less stable ($P < 5\%$) at 50% RH than at 80% RH, but it was lethal for monkeys. Atypical third-passage preparations showed maximal titers of about 10[7] MICLD50 at day 3 and did not induce cell lysis. This virus was not appreciably affected by changes in RH, although the overall viral recoveries appeared slightly decreased, and it was not lethal for monkeys.

TABLE 2. *Summary of changes in properties of yellow fever virus (Asibi strain) in HeLa cell cultures*

HeLa cell prepn	Maximal mouse ic log10 titer in HeLa cells	Day of maximal titer	Cytopathic effect[a]	Effect at 90°, RH[b]	Attenuation of virus for monkeys[c]
One passage	6.9-7.4	5	Negative	Resistant	Negative
	8 or >	5	Positive	Sensitive	Negative
Three passages	6.8-7.0	3	Negative	Resistant	Positive
	8 or >	3	Positive	Sensitive	Positive

[a] Cell rounding, increase in density, and detachment from glass.
[b] Effect of aerosolization on virus at 90°, relative humidity.
[c] By exposure to infected aerosols.

Comparison of Viral Properties After First and Third Passage

Some of the properties that were established for the various viral harvests appeared to be closely related to one another. An attempt to represent this is shown in Table 2 and by the following examples. (i) Virus grown after one passage in HeLa cells (approximately 10^7 MICLD$_{50}$ in 5 days) was virulent for monkeys. Conversely, virus grown in HeLa cells after three serial passages (10^8 or greater MICLD$_{50}$ in 3 days) was attenuated for monkeys. (ii) Virus that had shown a cytopathic effect was adversely affected by aerosolization at 50% RH, but virus that did not induce a CPE was unaffected. (iii) Viral harvests that failed to show maximal titers in excess of 10^7 MICLD$_{50}$, despite some degree of increased adaptation in HeLa cells (maximal titer at day 3), did not induce a cytopathic effect; harvests that contained titers of 10^8 MICLD$_{50}$ or greater induced a cytopathic effect in culture.

The results of these experiments also suggested that first-passage viral preparations were stabilized more easily than the third-passage preparations; 1 of 12 of the former populations behaved atypically, whereas 2 of 6 of the latter proved to be atypical. The atypical viral harvests shared properties with both the typical first- and third-passage preparations, and appeared, therefore, to represent intermediate viral populations. Additional work toward the further elucidation of such populations was indicated. Since multiple-passage preparations gave the greatest indication of genetic instability, we concentrated our experimental effort chiefly on characterizing the properties of viral harvests of this type.

FURTHER STUDIES ON VIRAL POPULATIONS AFTER ONE PASSAGE IN HeLa CELLS

Studies on virulence were expanded to include a comparison between rates of infectivity after administration of virus by the intraperitoneal (ip) as well as the respiratory route. To accomplish

TABLE 3. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after one passage in HeLa cells*

Dose (MICLD$_{50}$)	Lethality		Infectivity[a]	
	Resp[a]	ip[b]	Resp	ip
50	6/6[d]		--	
40		6/6		..
5.0	3/6		0/3[d]	
4.0		3/4		1/1
0.5	0/3		0/3	
0.4		3/6		2/3
0.05	NT[e]		--	
0.04		1/3		1/2

[a] Virus administered by respiratory route.
[b] Virus administered by intraperitoneal route.
[c] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[d] Number of monkeys affected per number treated with virus.
[e] Not tested.

this, graded doses of the same viral preparation isolated from HeLa cells were administered to monkeys by either route; survivors were challenged ip 21 days later with a multiple lethal dose of a mouse brain virus seed to determine whether the original administration of virus had subclinically infected the animals. The first series of tests with this experimental protocol was carried out with first-passage material to obtain base-line information with which to compare the third-passage preparations to be reported later.

Properties of Pre- and Postaerosolized Populations After One Passage

The results of these tests are represented in Table 3. Doses given by the respiratory route represent average values from several experiments. They show that doses of approximately 50 MICLD$_{50}$ given by either route were lethal for the

test monkeys. Doses of approximately 5 $MKLD_{50}$ of aerosolized virus were lethal for one-half of the exposed monkeys. A dose of 4 $MKLD_{50}$, however, was lethal for three of four monkeys injected by the ip route. Three of six monkeys succumbed when given a dose of 0.4 and one of three monkeys succumbed with a dose of 0.04 $MKLD_{50}$.

Animals that survived viral aerosols showed no evidence of having been infected. This was shown by the fact that three monkeys that failed to succumb to respiratory doses ranging from 1 to 10 $MKLD_{50}$ also failed to resist the ip challenge with a multiple lethal dose of suckling mouse brain virus. Thus, the dose that was necessary to infect appeared to be very close if not identical to the dose necessary to cause a lethal illness.

Two of three monkeys resisted a lethal challenge of virus after they survived a dose of 0.4 $MKLD_{50}$ given by the ip route; one of two monkeys that survived the ip dose of 0.04 $MKLD_{50}$ was not resistant. Thus, although there was some evidence that infection by the ip route was not invariably fatal with very low doses, it appears that the median lethal dose and the median infectious dose values obtained by the ip route were much closer to one another with first-passage virus than those obtained with multiple-passaged virus.

FURTHER STUDIES ON VIRAL POPULATIONS AFTER MULTIPLE PASSAGE IN HELA CELLS

Scheme of Tests

The next series of experiments was directed toward elucidating the characteristics of viral populations that arose after multiple serial passages in HeLa cells. Results of tests on two preparations obtained after three serial passages in HeLa cells and one other after seven passages will be presented. The following test scheme was devised for each viral harvest. After inoculation of the virus in culture, that is to say, during preparation of the third- or seventh-passage harvests, samples were obtained from the culture and titrated daily for 6 days postinoculation to establish the maximal titer and the time postinoculation of its occurrence. During that time, the cell sheet was examined microscopically for evidence of cell lysis. Both supernatant fluid and the cells were harvested at or near the time of maximal viral yields, and the material was frozen in glass ampoules. A few days later, a sample of this viral material was titrated intracerebrally in mice and by the ip route in monkeys. Then the preparation was aerosolized at 50 and 80% RH. The amount of virus that could be recovered in the aerosol and the extent to which the property of lethality for monkeys was decreased after the virus was airborne were determined. Values for the former were obtained by plotting recovery values at intervals during the 60-min period following aerosolization.

Virulence for monkeys was ascertained by exposing these animals at various intervals after rendering the agent airborne. Differences in dosage were obtained by exposing the monkeys to clouds of various ages, the older the cloud the smaller the dose. Impinger fluids were collected at intervals corresponding to those during which the monkeys were exposed, and these samples were injected ip into monkeys in such a manner that the same theoretical viral dose was administered to duplicate monkeys by either the respiratory or ip route. As in previous experiments, any monkey that survived the administration of the viral preparations by either route was challenged 21 days later with a multiple lethal dose of a mouse brain virus seed. Two uninfected control monkeys also were challenged in the same manner. The data presented in Table 4 show the results obtained with one third-passage preparation.

Properties of Pre- and Postaerosolized Third-Passage Viral Populations

In Table 4, the data are divided into two main sections. On the left are the results of injecting monkeys intraperitoneally with a third-passage HeLa cell preparation prior to its aerosolization. The most striking feature of these data is the lack of a clear-cut end point in the lethality pattern. Doses ranging from 3 to 3,000 $MKLD_{50}$ were lethal for one-half of the monkeys tested. All survivors were resistant to a multiple lethal dose of virus given 21 days later as an ip challenge. Two other monkeys that were originally injected with 0.3 $MKLD_{50}$ evidently were infected subclinically, since they also resisted the lethal challenge. The dose of 0.03 $MKLD_{50}$ apparently failed to infect the monkeys.

On the right side of Table 4 are the results of administering aerosolized virus to monkeys. Shown are the doses expressed as the number of $MKLD_{50}$, the RH employed during aerosolization of the virus, the lethality and infectivity resulting from exposing the monkeys to aerosolized virus and the lethality and infectivity of the impinger fluid into which the various viral doses were collected postaerosolization.

These data show once again that a clear-cut end point in the lethality pattern did not occur with either route; this was especially evident in monkeys exposed by the respiratory route. Moreover, there was no consistent difference in the incidence of lethality at 50 and 80% RH. This corresponds to the lack of any difference

TABLE 4. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after three serial passages in HeLa cells*

Preaerosolization				Postaerosolization					
	Intraperitoneal					Respiratory		Intraperitoneal	
Dose (mouse LD₅₀)	Leth[a]	Inf[b]	Dose (mouse LD₅₀)	RH (%)	Leth	Inf	Leth	Inf
3,000	1/2	1/1	2,304–915	50	1/2	1/1	0/2	2/2
300	1/2	1/1		80	1/2	1/1	1/2	1/1
30	1/2	1/1	210–54	50	1/2	1/1	2/2	—
3	1/2	1/1		80	2/2		1/2	1/1
0.3	0/2	2/2	12–3	50	2/2		1/2	1/1
0.03	0/1	0/1		80	1/2	1/1	0/2	2/2
			1–<1	50	0/2	0/2	0/2	1/2
				80	1/2	1/1	0/2	2/2

[a] Lethal for monkeys
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.

TABLE 5. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after three serial passages in HeLa cells*

Preaerosolization				Postaerosolization					
	Intraperitoneal					Respiratory		Intraperitoneal	
Dose (mouse LD₅₀)	Leth[a]	Inf[b]	Dose (mouse LD₅₀)	RH (%)	Leth	Inf	Leth	Inf
50,000	2/2		2,531–1,239	50	0/2	2/2	2/2	—
5,000	1/2	1/1		80	0/2	2/2	2/2	.
500	1/2	1/1	111–54	50	0/2	2/2	0/2	2/2
50	1/2	1/1		80	1/2	1/1	2/2	—
5	0/2	2/2	13–6	50	0/2	2/2	0/2	2/2
0.5	0/2	2/2		80	0/2	2/2	1/2	1/1
0.05	0/2	0/2	2–<1	50	0/2	0/2	0/2	0/2
				80	0/2	2/2	0/2	2/2

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.

between the per cent viral recoveries that were obtained at either humidity. Despite this, however, the RH effect might have been an influencing factor in the incidence of nonlethal infections in monkeys. For example, in the lowest dose range, no animals became infected when exposed to aerosols at 50% RH. At 80% RH, however, one of two monkeys succumbed, and the survivor was resistant to the lethal challenge. After injecting impinger fluids by the ip route, a slight increase may have occurred in the incidence of infectivity at 80% RH. Additional evidence of this is shown in data presented in Table 5.

In Table 5, the results of tests with a second third-passage preparation are shown. As with the previous findings, data on the left side of the table obtained with preaerosolized virus show once again the lack of a clear-cut end point in the lethality pattern. On the right side of the table, it can be seen, in contrast to the previous third-passage preparation, that no appreciable lethality was obtained by the respiratory route. Some lethality was found, however, after injecting the impinger fluids by the ip route; the lethality that occurred with the ip route appeared to be more pronounced at the 80% RH. Similarly, the incidence of infectivity was greater with very low doses of this viral preparation after it was aerosolized at 80% RH and administered by either route than when it was aerosolized at 50% RH.

TABLE 6. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after seven serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose (MICLD50)	Intraperitoneal		Dose (MICLD50)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
700,000	0/2[c]	2/2	2,108-1,198	50	0/2	2/2	0/2	2/2
70,000	NT[d]	NT		80	0/2	2/2	0/2	2/2
7,000	0/2	2/2	277-93	50	0/2	2/2	0/2	2/2
700	NT	NT		80	0/2	2/2	0/1	1/1
70	0/2	2/2	12-4	50	0/2	2/2	0/2	2/2
7	NT	NT		80	0/2	2/2	0/2	2/2
0.7	0/2	2/2	1-<1	50	0/2	0/2	0/1	1/1
0.07	NT	NT		80	0/2	2/2	0/2	2/2
0.007	0/2	0/2						

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.
[d] Not tested.

TABLE 7. *Number of MICLD50 necessary to produce infectivity with or without lethality after passage in HeLa cells*

Passage no.	Preaerosolization Intraperitoneal		RH (%)	Postaerosolization Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]		Leth	Inf	Leth	Inf
1st	0.04	0.04	50	5	5	NT	NT
			80	5	5	NT	NT
3rd (I)	3	0.3	50	3	3	3	<1
			80	<1	<1	210	<1
3rd (II)	50	0.5	50	>1,239	6	>1,239	<1
			80	210	<1	12	<1
7th	>700,000	0.7	50	>2,108	12	>2,108	1
			80	>1,098	<1	>1,098	<1

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.

Properties of Pre- and Postaerosolized Seventh-Passage Viral Population

The final test was carried out with a seventh-passage preparation, which resulted from a continuation of serial passages from the first of the third-passage harvests. Results of this test shown in Table 6 revealed that an increase in the attenuation of the virus had occurred; no lethality was encountered with either the pre- or post-aerosolized viral preparations. The incidence of infectivity, as shown by immunity to the lethal challenge, however, did not appear to have declined.

Comparison of Properties of Viral Populations After Multiple Passage in HeLa Cells

In Table 7, we have summarily compared the main points of interest of the three viral preparations. All of the values are expressed as the minimal number of MICLD50 that were shown to have induced either a lethal or nonlethal infection in monkeys. The data show that the pre- and post-aerosolized first-passage HeLa cell preparation proved to be a comparatively efficient inducer of lethality, although somewhat more virus appeared to be necessary to infect monkeys by the respiratory route than by the ip route. There did

not appear to be a significant difference between the viral response to either RH.

The first of the third-passage HeLa cell harvests showed a decreased level of lethality for monkeys when administered by either route. As the data in Table 4 had indicated previously, the assessment of the virulence of this preparation was greatly influenced by an apparent interference phenomenon that was expressed as a partial inability of this virus to cause lethality over a wide range of doses. Since the first-passage preparation did not display this phenomenon during its lethal effect in monkeys, it is tentatively concluded that the behavior of the third-passage harvest in this case indicates a weakening of the virulence character as a result of serial passage in HeLa cells. Information derived from other multiple-passage preparations clearly supports this view. The second of the third-passage preparations contained virus possessing a level of virulence that had declined to the extent that it failed to produce lethality by the respiratory route; moreover, it had markedly declined in its efficiency in producing lethal illness by the ip route. After seven passages, all traces of lethality for monkeys had vanished from the viral harvest. In all of the viral preparations, the loss in lethality was not accompanied by a loss in the capability of the virus to immunize the animals.

DISCUSSION AND SUMMARY

Manifestation of the viral "RH" marker was more subtle than expected in the two third-passage and seventh-passage preparations. In prior tests with this virus in our laboratory (4), the effect of RH was readily evident in experimental data showing that statistically significant decreases in viral recoveries were encountered upon aerosolization at 50% RH. This was similarly found to be the case with recovery values for airborne Colorado tick fever, vesicular stomatitis, neurovaccinia, and encephalomyocarditis viruses as reported by Watkins et al. (12). In the present results, the initial recovery values were not as high as those previously encountered in our laboratory, and it became necessary to use other criteria to demonstrate any difference that may have occurred as a result of aerosolization at 50 and 80% RH. In cases in which conditions were such that differences could be demonstrated, serially passed virus aerosolized at 50% RH was the least active.

The first of the third-passage preparations provided, at first glance, what might appear to be an exception to this. The dose of 210 MKLD$_{50}$ of the impinger fluid at 80% RH shown in Table 7 as that necessary to produce lethal illness in

monkeys by the ip route is higher than that necessary to produce lethal illness at 50% RH. Two factors appear to have been responsible for this. The first is that the dose of 210 MKLD$_{50}$ was inadvertently higher than that planned. On the basis of other data obtained under similar circumstances, the same clinical response would be expected to have been achieved with a much lower dose. Events such as these illustrate, perhaps, an important disadvantage in using the small number of animals that is usually necessary when monkeys must be employed.

In summary, the data presented in this paper clearly indicate that a pronounced loss of virulence rapidly occurred when yellow fever virus was serially passed in HeLa cells. By as early as the third passage, viral populations become demonstrably weakened in their ability to induce lethal illness in rhesus monkeys by either the ip or respiratory route. At this passage level, very high humidities were necessary to sustain even some semblance of lethality. Passage of the virus in cell culture, however, did not appreciably reduce the ability of the virus to induce an immunity in these animals.

From a genetic viewpoint, it is of considerable significance that the third-passage preparations were highly unstable. It is not surprising, therefore, that many of these viral populations possessed properties that varied to some extent from each other, not only in their degree of attenuation, but also in their response to 50% RH, and in their ability to cause a cytopathic effect in cell culture. In a previous publication (4), we noted that atypical first-passage and atypical third-passage viral populations that had been studied up to that time shared no properties with each other. This was unexpected, since both of these presumably represented viral forms that were intermediate to the virulent, aerosol-stable form and the attenuated aerosol-unstable form. Studies on third-passage populations that were recovered and studied since then have supported the view that these viral populations do represent truly intermediate forms. Furthermore, the data indicate that when a sufficient number of these unstable viral populations were examined, atypical intermediate forms that shared some of their properties with each other could be revealed. The genetic determinants for the viral properties that we have examined, therefore, are probably in close relationship to one another but obviously not linked.

The results of these studies raise the question of whether viral isolates of reduced virulence may be commonly acquired after passage in cell cultures. Viral mutants with either lowered

virulence or decreased stability, or both, might be easily selected in an in vitro system. The use of such mutant populations could reduce the danger of airborne contamination of laboratory workers, experimental animals, and other viral or cell culture materials. Venezuelan equine encephalomyelitis virus, another arbovirus, has been shown to lose its virulence in vivo as a result of its serial passage in vitro (3, 5). The question of whether this applies to other arboviruses can be determined only after an adequate number of suitable tests have been performed.

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Discussion

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Your supposition that a Dutch bacteriologist would have more experience of yellow fields of tulips than of yellow fever is absolutely correct. It is thus with great respect that I have read the careful and laborious experiments of Dr. Hearn on which he certainly is to be complimented.

Dr. Hearn described variations in yellow fever virus after passage in HeLa cells: (i) variation in growth rate, growth capacity, and the appearance of cytopathic effects; (ii) attenuation of virulence for monkeys; and (iii) variation in aerosol stability. These correlations are obvious and important. The HeLa cultures were passed at high multiplicity so that mixed populations were studied. Consequently, the bulk of the particles

in the atypical first passage (aTy 1) might well have lost the lethality for monkeys, but the population might still contain a few per cent of virulent particles. Again, the difference between the Ty 3 and aTy 3 might be due to interference or a von Magnus phenomenon. Thus, though the populations seem unstable, the variants might be quite stable genetically. Admittedly, yellow fever virology is very difficult, but, unless these variants are isolated from single plaques or passed at limiting dilutions, it is difficult to discuss these variations in terms of genetic markers. I sincerely hope that Dr. Hearn will find opportunity in the future to work in this direction.

The aerosol work is again of the highest level.

The monkeys were exposed to aerosols of different ages. The dose was expressed in terms of $MICLD_{50}$. (Let us hope this unit is constant before and after HeLa passage or aerosolization.) This means that, especially at lower doses, the monkeys receive a few viable particles and very many inactivated particles. Whether these inactivated particles still contain active ribonucleic acid is not known. It might well be then that the large number of inactivated particles in these experiments produced some kind of interference. The additional attenuation by aerosolization itself (Table 5) might also point in this direction. It would be interesting to know what happened when monkeys were exposed to various doses of aerosols of the same age.

All this, of course, detracts nothing from the fact that an important step has been taken in the direction of immunization with an avirulent yellow fever virus. In this connection, it would be important to know whether this virus could multiply in mosquitoes.

Aerosol Vaccination with Tetanus Toxoid

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INTRODUCTION

In recent years, the efficacy of aerosol vaccination against a variety of infectious diseases has been demonstrated in experimental animals and man. These diseases include Newcastle disease (14), distemper (11), tuberculosis (7, 20), tularemia (9), measles (15, 21, 25), Venezuelan equine encephalitis (29), and influenza (28, 30). Combined aerosol vaccination against Newcastle disease and infectious bronchitis (19) and tularemia and Venezuelan equine encephalitis (16) also has been effective in experimental animals. Russian investigators (1–3) have reported highly effective immunization of animals and man exposed aerogenically to dried living vaccines against anthrax, brucellosis, plague, and tularemia.

The vaccines employed in the above studies consisted mainly of viable attenuated organisms or their spores. Our studies were concerned with the immunological response following inhalation of a soluble antigen-tetanus toxoid. Tetanus toxoid is universally accepted as an effective immunizing antigen. Experience during World War II, when active immunization with this vaccine was first employed on a large scale for American and British forces, attests to its almost complete effectiveness in preventing tetanus (26). Although the initial conventional course of three doses provides long-lasting immunity (possibly for 10 years or more), reinforcing doses are invaluable for maintaining the titer of circulating antitoxin.

Studies in the USSR (23) have shown that aerogenic administration of diphtheria toxoid to experimental animals or man is an effective revaccination procedure after subcutaneous vaccination. A marked and prolonged rise in antitoxin titer was seen in guinea pigs and rabbits administered an aerosol booster of fluid toxoid 5.5 and 3 months after subcutaneous inoculation with aluminum hydroxide-adsorbed diphtheria toxoid (Table 1). The experiments were conducted in a 600-liter chamber. Purified concentrated toxoid with a potency of 2,100 units per ml was dispersed by means of a mechanical atomizer for 60 min.

By increasing the cloud concentration with toxoid electrically atomized with ultrasound in a 30-liter chamber, the Russian scientists reduced the period of aerosol exposure (23). A reduction in aerosol-exposure time from 30 min to 10 to 20 min had no effect upon the antibody response in guinea pigs administered an aerosol booster 3 to 4 months after subcutaneous inoculation with 6 units of adsorbed diphtheria toxoid (Table 2). A marked increase in antitoxin titer also was reported to occur in guinea pigs even after an exposure period of 1 to 2 min. A member of their laboratory staff was reported to have shown a marked rise in antitoxin titer after inhalation of diphtheria toxoid for 5 min.

Aerosol revaccination of children after primary subcutaneous inoculation also has been reported to be effective (24); undue systemic reactions were not observed. On the other hand, an allergic reaction was reported to have occurred in exposed adults. Concentrated dry toxoid given only as a primary dose or as a booster after subcutaneous vaccination was reported to produce increased antibody titers in rabbits and man (1).

AEROSOL VACCINATION WITH TETANUS TOXOID

Our current aerosol vaccination studies with fluid tetanus toxoid were performed with Hartley strain guinea pigs. Young adult guinea pigs of both sexes, weighing 300 to 470 g, were exposed to predetermined respiratory doses of fluid toxoid in groups of five to eight animals. The aerosol chamber consisted of an approximately 300-liter plastic hood installed within a biological safety hood. Purified concentrated fluid tetanus toxoid with a potency of 2,405 limit of flocculation (Lf) units per ml was sprayed with a University of

TABLE 1. *Reimmunization by inhalation of diphtheria toxoid with an exposure time of 60 min*[a]

Animals	No. of animals	Subcutaneous immunization		Interval between the first immunization and reimmunization	Mean antitoxin titers before the reimmunization	Mean antitoxin titers (units/ml) after reimmunization by inhalation at various times						
						Weeks					Months	
		Dose	Mean antitoxin-titers 1 month after subcutaneous inoculation			1	2	3	4	6	2	4
		units	units/ml	months	units/ml							
Guinea pigs	10	30	2.64	5.5	0.36	74	118	82	71		34	18.2
Rabbits	7	30	1.52	3	0.62	23.9	14.6		8.4	5		1.1

[a] Data from Muromstev et al. (23).

TABLE 2. *Reimmunization by inhalation of diphtheria toxoid with different exposure times*[a]

Group of animals	No. of guinea pigs	Subcutaneous inoculations		Interval between inoculations	Mean antitoxin titers before reimmunization	Exposure time for reimmunization by inhalation	Mean antitoxin titers (units/ml) at various times after reimmunization by inhalation					
							Weeks				Months	
		Dose	Mean antitoxin titers 1 month after immunization				1	2	3	4	2	4
		units	units/ml	months	units/ml	min						
First	8	6	1.4	3	0.83	30	71.2	88.7	101.5	62		19.1
Second	9	6	1.24	3	0.96	30	93.7	93.2	88.1	59.3		18.1
Third and fourth	11	6	1.16	4	0.97	10–20	83.2		114.2	66.6	27.5	

[a] Data from Muromstev et al. (23).

Chicago Toxicity Laboratory-type atomizer. The vaccine contained no preservatives and was obtained through the courtesy of Eli Lilly & Co. The fluid vaccine was fed to the atomizer by a 50-ml syringe, actuated with a motor-driven piston delivering 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of approximately 1 ft³/min.

A particle discrimination device, a British preimpinger, was used in the sampler system to obtain information on the particle diameter of fluid toxoid clouds. The preimpinger was fitted to a standard all-glass impinger (AGI-30) to select particles with diameters equal to or less than 5 μ in the collecting fluid. Physiological saline, the collecting fluid, was assayed for toxoid concentration by the flocculation test. Tetanus flocculating serum was furnished by the Division of Biologics Standards, National Institutes of Health. The respiratory dose was calculated as the product of the aerosol concentration, minute volume of respiration (27), and duration of exposure. Precalculated respiratory doses were administered by varying the duration of aerosol exposure.

TABLE 3. *Primary antibody response 5 weeks after aerosol or subcutaneous vaccination with fluid tetanus toxoid and survival 14 days after subcutaneous challenge with tetanus toxin*

Primary vaccination		Hemagglutination titer		Survival after challenge	
Route	Dose (Lf)	Geometric mean	Range	Survivors/total	Per cent
Respiratory	4[a]	24	<10–320	1/7	14.2
	10[a]	79	<10–1,950	4/13	30.7
	15[a]	200	10–640	7/13	53.8
	19[a]	336	<10–2,560	3/8	37.5
	32[a]	72	<10–1,280	4/7	57.1
Subcutaneous	9	1,158	300–4,800	6/6	100.0
	15	4,609	600–19,200	6/6	100.0

[a] Calculated respiratory dose (±1 Lf); ≤5 μ particles.

PRIMARY AEROSOL VACCINATION AND CHALLENGE

The primary antibody response of guinea pigs after inhalation of various doses of aerosolized fluid tetanus toxoid (particles ≤5 μ) and survival data taken after subcutaneous toxic challenge are

TABLE 4. *Serum hemagglutination titer and survival of aerosol-vaccinated guinea pigs after subcutaneous challenge with tetanus toxin*

Hemagglutination titer	Survival after challenge	
	Survivors/total	Per cent
<160	3/29	10.3
>160-<640	5/8	62.5
>640	11/11	100.0

summarized in Table 3. The duration of aerosol exposure varied from 20 to 80 min. Control animals were vaccinated with fluid toxoid by the subcutaneous route. Blood samples for serological analysis were obtained by cardiac puncture 5 weeks after vaccination, and the guinea pigs were challenged 1 week later with 10 guinea pig MLD of tetanus toxin administered by the subcutaneous route. The results demonstrated that mean serum antibody titers, measured by the passive hemagglutination (HA) procedure (31), were lower after inhalation of toxoid than after subcutaneous inoculation with comparable doses of fluid toxoid.

A mean serum HA titer of approximately 526 or greater was protective against subcutaneous challenge with 10 MLD of tetanus toxin. The relationship between HA titer of aerosol-vaccinated guinea pigs and survival after toxic challenge is summarized in Table 4. Guinea pigs

vaccinated by the subcutaneous route had titers ranging from 300 to 19,200 and did not succumb to the toxic challenge dose (Table 3).

One possible contributing factor to the range of antibody responses following aerosol vaccination (Table 3) may be variations in the breathing capacity or pattern of individual guinea pigs. Since minute volume of respiration was not determined for the individual guinea pigs and is based on a reference value, variations may be expected to occur in the actual inhaled dose. It is unlikely, however, that the irregularities in antibody response could be attributed wholly to differences in breathing. The response seen with respiratory doses of 15 and 19 Lf units certainly should have been eliminated at 32 Lf units if this were the only explanation.

The position of the animals in the aerosol chamber in relation to the atomizer did not appear to influence the antibody response. Cloud samples, obtained throughout the aerosol exposure period, were quite uniform in toxoid concentration, as determined by the flocculation test. Other factors which can influence the efficiency of aerosol immunization are discussed below.

AEROSOL REVACCINATION

Results from experiments to evaluate the secondary antibody response following inhalation of atomized fluid tetanus toxoid are summarized in Tables 5 and 6. Guinea pigs were bled for HA

TABLE 5. *Secondary antibody response with aerosol booster 6 weeks after inhalation of fluid tetanus toxoid*

Respiratory dose (Lf)		No. of guinea pigs	Determination	Hemagglutination titer			
Primary	Secondary			Prebooster[a]	Weeks after booster		
					1	5	10
8	6	14	Mean[b]	47	3,898	5,099	953
			Range	<10-1,280	10-256,000	<10-128,000	10-48,000
6	14	7	Mean[b]	176	9,696	9,729	3,772
			Range	10-1,920	<10-192,000	1,280-384,000	400-32,000
14	5	7	Mean[b]	732	188,988	31,051	7,463
			Range	10-12,800	12,800-1,024,000	4,000-192,000	2,000-48,000
					Weeks after primary		
					7	11	16
11	0	4	Mean[b]	708	774	235	132
			Range	160-2,560	<10-2,400	<10-9,600	<10-1,920
21	0	4	Mean[b]	80	293	280	140
			Range	<10-5,120	<10-25,600	<10-16,000	<10-4,000

[a] At 5 weeks after primary vaccination.
[b] Geometric mean.

Table 6. *Secondary antibody response with aerosol booster 6 weeks after subcutaneous vaccination with fluid or alum-precipitated tetanus toxoid*

Subcutaneous vaccination	Dose (Lf)	Respiratory booster dose (Lf)	No. of guinea pigs	Determination	Hemagglutination titer			
					Prebooster[a]	Weeks after booster		
						1	5	10
Fluid toxoid	7.5	5	3	Mean[b]	29,081	133,126	146,520	66,463
				Range	6,400–102,400	96,000–192,000	64,000–384,000	24,000–192,000
		9	11	Mean[b]	5,335	101,640	74,965	24,490
				Range	1,280–25,600	32,000–384,000	48,000–128,000	8,000–64,000
		0	5	Mean[b]	10,808	4,095-	8,386	6,553
				Range	6,000–16,000	2,000–12,000	3,000–48,000	400–25,600
Alum-precipitated toxoid	7.5	3	4	Mean[b]	36,472	627,070	503,560	524,280
				Range	12,000–192,000	384,000–1,024,000	256,000–768,000	256,000–1,024,000
		10	11	Mean[b]	52,050	694,130	296,410	317,490
				Range	8,000–32,000	96,000–3,072,000	32,000–1,024,000	64,000–768,000
		0	8	Mean[b]	6,586	10,004	11,272	8,086[d]
				Range	1,000–16,000	2,000–32,000	2,400–25,600	2,400–16,000

[a] At 5 weeks after primary vaccination.
[b] Geometric mean.
[c] At weeks after primary vaccination.
[d] One guinea pig lost from group.
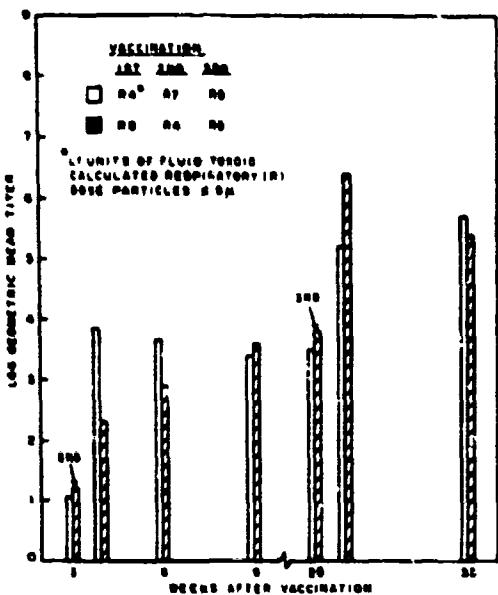
FIG. 1. *Serum hemagglutinating antibody response in guinea pigs after vaccination series with fluid tetanus toxoid administered by the respiratory route.*


FIG. 2. *Serum hemagglutinating antibody response in guinea pigs after vaccination series with fluid tetanus toxoid administered by combinations of subcutaneous and respiratory routes.*

antibody 5 weeks after the primary vaccination and 1, 5, and 10 weeks after the aerosol booster.

Aerosol revaccination of guinea pigs sensitized initially by the respiratory route resulted in an enhancement of the HA antibody response (Table 5). The range of titers indicates, however, that adequate sensitization was not achieved in all animals. A few guinea pigs showed essentially no serum antibody response after either primary or secondary aerosol stimulation. However, when sensitization was adequate, a marked enhancement of the antibody response occurred in all guinea pigs after secondary aerosol exposure to tetanus toxoid. This is seen with animals administered a primary respiratory dose of 14 Lf units, followed by an aerosol booster with 5 Lf units of toxoid. The control guinea pigs received respiratory doses approximately equal to the total doses employed in the revaccination studies.

Aerosol revaccination of guinea pigs inoculated initially by the subcutaneous route with a standard dose of either fluid toxoid or alum-precipitated toxoid (Table 6) was more effective than respiratory-respiratory vaccination. In this case, adequate sensitization of all guinea pigs was achieved prior to the aerosol booster. The response was superior in animals receiving the alum-precipitated toxoid preparation as the primary sensitizing dose.

In another experiment, the HA antibody re-

sponse was followed over a period of approximately 8 months, with small groups of guinea pigs revaccinated by the aerosol route after a combination of vaccine treatments with fluid tetanus toxoid. The mean serum HA antibody response was measured at various intervals with pairs of guinea pigs revaccinated 3 and 28 weeks after the primary vaccination.

The mean serum HA antibody response after a vaccination series of three respiratory doses of fluid toxoid is summarized in Fig. 1. Although the response was minimal 3 weeks after primary inhalation of 4 or 8 Lf units of fluid toxoid, revaccination by the respiratory route resulted in a marked enhancement of the antibody response after secondary and tertiary stimulations. The mean titer prior to the tertiary respiratory booster was 4,280 for both groups. After tertiary inhalation of vaccine, the titers of the individual guinea pigs were >192,000. The total calculated respiratory dose for both groups was approximately 20 Lf units. A subsequent challenge to one guinea pig from each subgroup with 10 MLD of tetanus toxin administered by the subcutaneous route did not result in signs of tetanus or death during a 14-day observation period. Unfortunately, one guinea pig from each group died from the intracardial bleeding procedure prior to the toxic challenge.

Figure 2 summarizes the mean serum HA antibody response after respiratory revaccination of guinea pigs initially vaccinated by the subcutaneous-subcutaneous or subcutaneous-respiratory routes with fluid toxoid. The total dose administered in these experiments was approximately 23 Lf units. The antibody response was enhanced in all three groups of animals after secondary and tertiary stimulations by a combination of subcutaneous and respiratory treatments.

Two groups of guinea pigs administered a vaccination series of two subcutaneous doses followed by a respiratory dose showed comparable responses, although the subcutaneous doses were given in different combinations. Prior to inhalation of the vaccine, the mean HA titer of both groups was 50,904. After the respiratory booster, the individual titers of the guinea pigs were >512,000.

Guinea pigs receiving the subcutaneous-respiratory vaccination series showed only a transient increase in titer after the respiratory booster. The titer essentially dropped to its prebooster level by the 6th week and then increased to 25,600 by the 25th week after the secondary booster. This "delayed response," which was also evident to a lesser degree in animals vaccinated with similar doses by the subcutaneous-subcutaneous routes, has been previously reported in guinea pigs after two injections of diphtheria toxoid (5). After the tertiary aerosol booster, the individual titers of the guinea pigs were >256,000.

The long-term antibody response therefore was comparable in all three groups. None of the guinea pigs responded during a 14-day observation period to 10 MLD of tetanus toxin administered by the subcutaneous route.

PARTICLE-SIZE RELATIONSHIP

As reviewed by Langmuir (18), the recognition that respiratory deposition and retention of inhaled particles vary with aerodynamic particle size is perhaps the most significant finding in aerobiology during the past two decades.

Recent studies by several investigators (6, 13, 22) indicate that the critical diameter of particles for deep lung deposition and retention is 1 to 5 μ. Hatch (13) reported that deposition of inhaled particles in the respiratory spaces of the lung occurs with the highest probability with particles 1 to 2 μ in diameter (gravity settlement) and with those in the submicroscopic region below 0.2 μ (precipitation by diffusion). Particles larger than 5 μ may not reach the respiratory bronchioles and alveoli; most between 0.25 and 0.5 μ may be exhaled.

Table 7. *Absolute recovery of sodium fluorescein-tetanus toxoid cloud particles at various relative humidities (RH)*

Impactor particle size	Mean recovery ($\mu g \pm$ SD)		
	61-70% RH	71-80% RH	81-90% RH
μ			
16	5.8 ± 0.4	6.5 ± 0.3	5.v ± 1.3
8	17.7 ± 3.2	19.5 ± 1.3	17.6 ± 4.0
4	9.1 ± 3.0	8.9 ± 0.5	8.5 ± 1.7
2	2.3 ± 0.4	3.1 ± 0.4	2.6 ± 0.6
1	2.7 ± 1.0	1.6 ± 0.3	1.5 ± 0.2
0.5	2.4 ± 1.0	0.5 ± 0.3	0.5 ± 0.1
0.25	2.9 ± 1.2	0.7 ± 0.3	0.2 ± 0.1
Total	42.9 ± 5.0	40.8 ± 1.8	36.8 ± 5.7
No. of trials	4	7	5

The importance of aerosol particle size to airborne pathogenesis is illustrated by the studies reported by Goodlow and Leonard (10). These investigators demonstrated a relationship between particle diameter and LD_{50} in guinea pigs and monkeys exposed to aerosols of *Pasteurella tularensis*. The LD_{50} for guinea pigs increased by almost 4 logs as the particle diameter increased from 1 to 12 μ.

The critical parameters for quantitative characterization of aerosols have been recently summarized by Goodlow and Leonard (10) and Wolfe (32). Basic parameters such as particle-size distribution, physical and biological decay rates, age of aerosol, and technique of aerosolization are critical not only in experimental airborne infection but also in aerosol immunization.

The particle-size distribution of tetanus toxoid clouds was studied with a Battelle miniature cascade impactor (BMCI), with sodium fluorescein as a physical tracer. Fluorometric assay of each stage of the BMCI was performed with a Coleman 12C electronic photofluorometer. Tables 7 and 8 summarize the absolute recovery and the cumulative recovery, respectively, of sodium fluorescein from each stage of the impactor during 16 trials conducted with sodium fluorescein-tetanus toxoid clouds at various relative humidities. The sensitivity of the particle-sizing device did not allow measurement of particles in the submicroscopic region below 0.25 μ. Also, since the sensitivity of relative humidity measurements can vary by approximately 5%, the data are grouped arbitrarily into three humidity ranges.

The data presented in Table 7, expressed as the mean absolute recovery of sodium fluorescein, suggest higher recoveries of particles less than

TABLE 8. *Cumulative recovery of sodium fluorescein-tetanus toxoid cloud particles at various relative humidities (RH)*

Impactor particle size	Determination	Recovery (%)		
		61-70% RH	71-80% RH	81-90% RH
16	Mean	100.0	100.0	100.0
8	Mean	84.2	83.8	82.4
	Range*	79.0-93.4	78.3 89.3	75.9 88.9
4	Mean	45.8	36.1	35.6
	Range*	39.9-51.7	31.6-40.6	30.3-40.9
2	Mean	26.0	14.1	12.7
	Range*	17.3-34.5	7.7-20.9	5.1-20.3
1	Mean	20.4	6.5	5.7
	Range*	13.1-27.7	0.9-12.1	0.0-12.3
0.5	Mean	13.4	2.7	1.7
	Range*	8.7-18.1	0.0-6.2	0.0-5.9
0.25	Mean	8.7	1.3	0.4
	Range*	3.8-9.6	0.0-3.7	0.0-3.0

*Values are 95% confidence limits for the estimate of the mean, with variance pooled across humidities for each particle size.

2 μ in diameter at humidities below 70%. The same data, expressed as cumulative per cent recovery in Table 8, indicate a similar trend toward recovery of smaller particles at humidities below 70%. Since only a few trials were performed for each humidity range, the data are presented only as a preliminary conjecture for possibly relating particle-size distribution of aerosolized fluid tetanus toxoid to degree of immunological response.

In a preliminary communication (33), we reported that, after inhalation of 9 or 15 Lf units of toxoid, three of eight and six of seven guinea pigs, respectively, survived a challenge of 10 MLD of tetanus toxin given subcutaneously 6 weeks after the primary vaccination. The mean HA titers were 71 and 278 for the 9- and 15-Lf doses, respectively, 5 weeks after vaccination. The primary antibody responses at 5 weeks from these and subsequent aerosol immunizations are summarized according to relative humidities above and below 70% in Table 9. The data suggest a possible relationship between particle-size distribution of toxoid as a function of relative humidity and degree of immunological response. Until additional trials are conducted at lower humidities, however, the results are not conclusive.

One of the critical parameters that requires further investigation is the antigenic stability of aerosolized tetanus toxoid at various relative humidity ranges. The problem of aerosol decay is less acute in a continuously generated aerosol than in a static aerosol. As indicated earlier,

TABLE 9. *Relationship between relative humidities above and below 70% and primary antibody responses 5 weeks after inhalation of various doses of fluid tetanus toxoid*

Relative humidity range	Calculated respiratory dose (Lf)	No. of guinea pigs	Hemagglutination titer		
			Geometric mean	Range	Per cent >1:20
71-80	9	12	37.7	<10-1,280	25.0
	15	17	534	<10-12,800	58.8
	20	5	199	<10-5,120	40.0
61-70	9	24	171	<10-2,560	41.7
	15	15	75	<10-960	40.0
	20	15	561	<10-5,120	86.7

* Calculated respiratory dose (±2 Lf units).

TABLE 10. *Antigenicity of aerosolized tetanus toxoid administered subcutaneously*

Fluid tetanus toxoid	Subcutaneous dose (Lf)	No. of guinea pigs	Geometric mean hemagglutination titer		
			1 week*	3 weeks	6 weeks
Aerosol[b]	7.5	3	<10	1,280	8,776
Stock	7.5	3	<10	1,846	7,241[c]
Stock	5.0	3	<10	1,016	5,120
Stock	2.5	3	<10	528	1,099

* Weeks after primary vaccination.
[b] AGI 30 sample; ≤5 μ particles; collected in physiological saline.
[c] One guinea pig lost from group.

tetanus toxoid clouds were quite uniform in their flocculating capacity throughout aerosol exposure. Mean nominal per cent recoveries of toxoid as determined from AGI-30 samplers were 7.6 and 6.6% for humidity ranges from 61 to 70% and 71 to 80%, respectively. The loss of tetanus toxoid immunogenicity was not significant when guinea pigs were inoculated subcutaneously with aerosolized toxoid material collected at a relative humidity of approximately 85%. The antibody response was comparable to that achieved in guinea pigs inoculated subcutaneously with equivalent concentrations of stock fluid toxoid (Table 10).

Many other factors should be considered in relating aerodynamic particle size to degree of penetration and retention in the lungs. The breathing pattern and capacity of the experimental animal at the time of aerosol exposure may be influenced by such conditions as excitability and stress. The physical and chemical nature of

the aerosolized material and its composition considerably influence its passage down the respiratory tree during inspiration. The interaction of small and large particles in a heterogeneous aerosol may determine the particle-size diameter during the passage of an aerosol through the respiratory tract.

Since air in the lungs is nearly saturated with water vapor, particles sensitive to humidity may increase in size immediately after reaching the nostrils or trachea, where the relative humidity is above 90% (8). The activity or function of the mucociliary system in the upper respiratory tract must be considered in determining the fate of an inhaled foreign particle (4, 6). However, in contrast to a particulate substance, soluble material would be expected to be absorbed rapidly from most surfaces within the respiratory tract, thereby obviating the necessity of other physiological processes (6). These and other factors, such as electrostatic charge on the particle and on the body of the experimental animal (22), play a vital role in the effectiveness of penetration and retention of particles in the respiratory tract.

The preliminary data presented, from studies still in progress, serve only to emphasize the potential of immunization by inhalation of a nonviable soluble antigen. That effective primary immunization of guinea pigs can be achieved by aerosol vaccination is evident. An aerosol booster after either primary respiratory or primary subcutaneous vaccination appears to be an effective reimmunization procedure. Attempts to relate discrepancies in the immune response to particle-size distribution of aerosolized fluid tetanus toxoid resulted in data that, although not conclusive, provide a working basis for more definitive studies relating aerodynamic particle size to degree of immunological response. As indicated by Lamanna (17), definitive studies are lacking on the mechanisms of permeability of respiratory tract tissue to substances of high molecular weight.

Of obvious concern during the guinea pig immunization studies was the possible induction of hypersensitivity in the animals by administration of toxoid by the respiratory route. The guinea pig is especially prone to development of the hypersensitive state. Untoward reactions were not observed after primary aerosol vaccination with fluid toxoid or after secondary aerosol treatment in combination with aerosol or subcutaneous vaccination procedures. However, guinea pigs receiving two subcutaneous doses of alum-precipitated toxoid at an interval of 3 weeks, followed by a tertiary aerosol booster

with fluid toxoid 26 weeks later, developed signs resembling anaphylaxis, resulting in death of some of the animals. The important consideration here which requires closer and further evaluation is the dosage, interval, and frequency of vaccinations

SUMMARY AND CONCLUSION

The method of aerosol vaccination might confer superior protection against pathogenic microorganisms whose natural portal of entry is the respiratory tract (29). Although inhalation of tetanus toxin per se represents an unnatural circumstance, except as an intentional act of war (17), aerosol vaccination with tetanus toxoid, and possibly other soluble antigens derived from pathogenic microorganisms, may provide a rapid and painless method of immunization.

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Discussion

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It was clearly shown by Dr. Yamashiroya that inhalation of purified tetanus toxoid will induce a well-marked primary immune response in the guinea pig, and that the secondary response following toxoid inhalation by a primed animal is even better. Dr. Yamashiroya's results seem to indicate that the relative humidity, by affecting the particle size distribution of the aerosol, may influence its immunogenic effectivity. It should be observed that the size distribution of droplet nuclei is mainly determined by the concentration of the spray solution, and it would seem worthwhile to study comparatively the immune responses of guinea pigs in Dr. Yamashiroya's experimental model by varying the concentration of the sprayed toxoid solution inversely to the period of exposure.

Site of deposition, in relation to particle size, is generally assumed to be an important factor in determining the infective or immunizing effects of inhaled aerosols (3, 5). However, various other factors, in addition to influences on the retention and site of deposition of the particles, as properly referred to by Dr. Yamashiroya, may affect the mechanisms underlying the immune response after the inhalation of antigens.

In a study on aerosol immunization against tetanus, we have investigated the effect of adding bacterial adjuvants to the toxoid spray solution. Groups of 20 mice were exposed in a modified Henderson apparatus (7) to the immunizing aerosols, sprayed with a Collison nebulizer from solutions containing 7,000 to 10,000 limit of flocculation (Lf) units of toxoid per milliliter. It should be admitted that, in comparison with guinea pigs, mice are less suitable for immunization studies with unabsorbed fluid tetanus toxoid, the results being rather irreproducible (1). A further disadvantage is caused by the fact that it is hardly feasible to follow the serum antibody level over a prolonged period of time in individual animals, as was done by Dr. Yamashiroya in guinea pigs. Nonetheless, in our experimental model, we were able to demonstrate that the primary immune response following toxoid inhalation is strikingly enhanced by the addition of certain bacterial adjuvants.

Immune responses were evaluated, 3 weeks after immunization, by subcutaneous challenge with 50 mouse LD₅₀ of toxin, and by antitoxin titrations in the pooled sera of small groups of animals. All sera were titrated in vitro by the indirect hemagglutination (HA) technique, as de-

scribed by Stavitsky (10); in addition, a number of sera were also titrated in vivo by the mouse protection test on a 0.001 antitoxin unit (AU) level. Toxoid aerosols were sampled in liquid impingers

TABLE 1. *Primary immune response of mice 3 weeks after administration of plain tetanus toxoid by aerosol or by subcutaneous injection*

Route of administration	Dose (Lf)	Survivors 5 days after challenge[a]	Antitoxic serum titer (hemagglutination)[b]
Aerosol	10-12	0/20	ND
	20-24	1/20	ND
	40-48	1/16	<10
Subcutaneous injection	0.5	11/20	128
	1.0	20/20	ND
	2.0	20/20	320

[a] Number of survivors/number challenged.
[b] Hemagglutination titrations of pooled sera of four animals from each group; ND, not determined.

TABLE 2. *Immune response of mice 3 weeks after repeated administration of plain tetanus toxoid by various combinations of inhalation and subcutaneous injection*

First dose (Lf)		Second dose (Lf)[a]		Survivors 5 days after challenge[b]	HA antitoxic serum titer[c]
10-12	(R)	10-12	(R)	1/20	ND
0.5	(I)	10-12	(R)	16/16	4,000
0.5	(I)	0.5	(I)	16/16	4,000
10-12	(R)	—		0/20	ND
0.5	(I)	—		11/20	128

[a] Doses given 3 weeks apart.
[b] Number of survivors/number challenged.
[c] HA titrations of pooled sera of four animals from each group. ND, not determined; (R), given by the respiratory route; (I), given by injection.

and evaluated by indirect hemagglutination inhibition tests (10). Inhalation doses were estimated from cloud sample assays, periods of exposure, and mouse respiratory volumes as defined by Guyton (4).

The results of a series of experiments with

aerosols of plain toxoid are presented in Table 1. In preliminary tests, the 50% protective dose by subcutaneous injection had been found to be in the order of 1 Lf; therefore, control animals were given toxoid by the subcutaneous route in doses ranging from 0.5 to 2.0 Lf. The figures clearly demonstrate that under these experimental conditions almost no immune response is elicited in mice by a single inhalation of plain toxoid at dosages up to 40 Lf. On the other hand, a single injection of a dose as small as 0.5 Lf induces partial protection.

Results obtained with aerosol treatment in two doses given 3 weeks apart are presented in Table 2. Also shown are the effects of a booster treatment by aerosol in comparison with that of an injection. It is seen that inhalation of plain toxoid on two occasions did not induce a better

TABLE 3. *Immune response of mice 3 weeks after single or repeated administration of a mixture of tetanus toxoid and Bordetella pertussis cells by aerosol or by subcutaneous injection*

First dose (Lf)	Second dose (Lf)[a]	Survivors 5 days after challenge[b]	Antitoxic serum titer[c]	
			Hemagglutination	Mouse test
16 (R)	—	15/16	128	ND
0.5 (I)	—	13/16	ND	ND
16 (R)	16 (R)	16/16	16,000	1.15 AU
0.5 (I)	0.5 (I)	16/16	8,000	0.80 AU

[a] Doses given 3 weeks apart.
[b] Number of survivors/number challenged.
[c] Titrations of pooled sera of four animals from each group; ND, not determined (R), given by the respiratory route; (I), given by injection.

immune response than did a single inhalation. However, when given as a booster to mice already primed by injection, the toxoid inhalation provoked a marked anamnestic reaction, similar to that induced by a booster injection.

The strong potentiating effect of a bacterial adjuvant on the immunizing activity of inhaled toxoid is illustrated by the data shown in Table 3. Killed *Bordetella pertussis*, an effective adjuvant (9), was mixed with the plain toxoid solution to a concentration of approximately 4 × 10¹⁰ organisms per milliliter. Control animals received adequate doses of the same mixtures by injection. Since no figures are available for the retention of the components of the inhaled mixture, a comparison of the dosages used for immunization by the respiratory route and by injection cannot be made. Nonetheless, it is evident that toxoid given by aerosol together with *B. pertussis* cells conferred a significant degree of immunity (compare Tables 1 and 2), and that, at the given doses, both the primary and secondary immune responses were not inferior to those following injection.

It has been shown by others (2) that, in stimulating the antibody response to protein antigens, *B. pertussis* extracts may be as active as whole-cell preparations. To investigate the effect of such an extract on the immunogenicity of the inhaled toxoid, additional experiments were carried out. Two fractions, which had been derived from *B. pertussis* in the procedure for the preparation of a soluble pertussis vaccine at the National Institute of Public Health, Utrecht (6), were tested. According to data provided by the laboratory for vaccine production of the National Institute of Public Health, one of these

TABLE 4. *Immune response of mice 3 weeks after single or repeated administration of mixtures of tetanus toxoid and Bordetella pertussis preparations by aerosol*

Mixture	First dose (Lf)	Second dose (Lf)[a]	Survivors 5 days after challenge[b]	Antitoxic serum titer[c]	
				Hemagglutination	Mouse test
T + C	8-10	—	20/20	ND	ND
T + I	8-10	—	20/20	ND	ND
T + II	8-10	—	16/20	ND	ND
T + C	8-10	8-10	10/10	320,000	32 AU
T + I	8-10	8-10	10/10	160,000	32 AU
T + II	8-10	8-10	10/10	80,000	8 AU
T Pl.	16-20	—	0/10	ND	ND
T Pl.	16-20	16-20	0/20	ND	ND

[a] Doses given 3 weeks apart.
[b] Number of survivors/number challenged.
[c] Titrations of pooled sera of 10 animals from each group; ND, not determined; T + C, toxoid + whole cells; T + I, toxoid + extract; T + II, toxoid + cell wall debris; T Pl., plain toxoid.

fractions, the soluble extract, exhibited nearly all the biological activity contained in the original cell suspension, in terms of immunizing potency and histamine-sensitizing factor. In the other fraction, consisting of cell wall debris, no such biological activity could be discovered. As can be seen from the data summarized in Table 4, the extract did indeed show an adjuvant activity similar to that of the whole-cell preparation. However, the cell wall debris also appeared to be an active adjuvant. A further analysis of these fractions will be needed to determine the factors responsible for the observed adjuvant effects.

From these experiments, as compared with those of Dr. Yamashiroya, it may be concluded that mice show a much weaker immune response to inhaled plain tetanus toxoid than do guinea pigs. This may be associated with a lower degree of cellular reactions, in terms of phagocytic activity and lymphoid hyperplasia. Such a difference between mice and guinea pigs has also been observed by Henderson in his studies on the infectious processes developing after inhalation of certain bacterial pathogens (8). The highly potentiating effect of *B. pertussis* preparations in aerosol vaccination of mice has been clearly demonstrated in our experimental model. The histological changes in the respiratory tract and adjacent lymphoid tissues of the mouse, associated with this adjuvant effect, are being studied.

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Aerosol Infection of Monkeys with
Rickettsia rickettsii

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INTRODUCTION

There are many infectious diseases which are not transmitted via the air-borne route. Such is the case with Rocky Mountain spotted fever. Studies in both man and animals have clearly established that transmission occurs through the bite of an infected tick and less frequently through abrasions in the skin contaminated with infected tick feces or tissue juices.

Recent studies indicate that aerosols of arthropod organisms, under certain conditions, can lead to disease (10, 20, 21, 24, 25). To our knowledge, human infection due to aerosol exposure to *Rickettsia rickettsii* has not been documented. Rocky Mountain spotted fever of man has characteristic features which are remarkably similar to those of monkeys injected with infectious material (1, 27). It remained for experimental studies to determine whether the portal of entry of the infectious agent alters the characteristics of the disease, its pathology, and the response to prophylactic and therapeutic measures. It is the purpose of this paper to describe the results observed in monkeys challenged with *R. rickettsii* by the respiratory route.

RESPONSE OF NORMAL MONKEYS TO PRIMARY CHALLENGE

Clinical Aspects

During the course of these studies (17), rhesus and cynomolgus monkeys were exposed in a model 3 Henderson apparatus (7, 15) to aerosols of the R1 strain (13) of *R. rickettsii* propagated in the yolk sac of developing chick embryos (23). Assuming that the respiratory volume was about 1 liter (4) and that about 50% of the particles were retained (6), the monkeys received challenges varying from 1.5 to 9,000 yolk sac LD_{50}. Clinically obvious disease was observed in 56 of 60 (93.3%) rhesus monkeys, and 42 of 56 (75.0%) died of specific infection 7 to 24 days postchallenge; 38 of 56 (67.9%) exhibited rash.

Clinical findings in the 56 rhesus monkeys that became ill after challenge are summarized in Table 1. Fever was observed in all; rectal temperatures of 105 to 106 F (40.6 to 41.1 C) were common. Monkeys usually became febrile 5 to 7 days after exposure and 1 to 2 days before appearance of other symptoms. Monkeys usually survived if their fever did not persist for more than 2 or 3 days.

Lethargy, anorexia, and weakness were first observed from the 6th to 9th postexposure day (PED). Monkeys sat quietly with heads hanging and arms clasped about the body and were noticeably weak when removed from the cage. In monkeys that eventually died, symptoms became progressively worse during the next few days. Animals would not respond to stimuli, did not resist handling, and could not rise from a lying position. Monkeys usually were in coma for 12 to 24 hr prior to death. Terminally, the pupils were dilated and nonreactive, and patellar reflexes were absent. Respirations were usually deep, abdominal, and at the rate of 4 to 6 per min. The body was cold on palpation, and the rectal temperature was less than 100 F (37.8 C).

TABLE 1. *Clinical findings in 56 rhesus monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Clinical finding[b]	No. of mon-keys	Per cent
Fever	56	100.0
Chills or tremors	8	14.3
Lethargy, anorexia, weakness	56	100.0
Heart sounds less intense and muffled	53	94.6
Respiratory signs[c]	12	21.4
Dehydration	9	16.1
Diarrhea	3	5.4
Rash	38	67.9
Peripheral necrosis	9	16.1

[a] Data from Saslaw et al. (17).

[b] Delirium, 2; pruritis (general), 2; epistaxis, 1; hemorrhagic rhinitis, 2; purulent nasal exudate, 1; serous conjunctivitis, 1; suppurative conjunctivitis, 1; suppurative inflammation of rash, 1.

[c] Dyspnea, 10; râles, 4; friction rubs, 3; percussion dullness, 2; coughing, 1; sneezing, 1.

TABLE 2. *Distribution and time of appearance of rash in 38 rhesus monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Site	No. of mon-keys	Per cent	Appearance (mean post exposure day)
Thighs	27	71.5	11.1
Legs, below knees	18	47.4	11.5
Feet	5	13.2	15.4
Face	22	57.9	11.7
Ears	24	63.2	12.7
Arms	13	34.2	11.7
Hands	4	10.5	15.3
Genitalia	14	36.8	11.9
Pubic, perineal, perianal areas	9	23.7	11.7
Back	6	15.8	10.9
Tail	7	18.4	11.1
Total	38	100.0	11.1

[a] Data from Saslaw et al. (17).

The earlie t death occurred on the 7th PED, and one monkey died on the 8th day; 29 of 42 (69.1%) died on the 9th through 12th PED, and 11 of 42 (26.2%) survived beyond the 12th day. The median survival time was 11 days, and the mean, 12.3 days.

The course of the illness in 14 surviving monkeys was highly variable. One monkey became acutely ill and was comatose for about 24 hr, but eventually recovered, whereas three monkeys showed only mild lethargy and anorexia. Intermediate gradations of disease were observed in the remaining 10 animals. Surviving monkeys usually were febrile for 5 to 7 days and ill for 7 to 10 days.

Heart sounds became less intense and muffled with, or shortly after, onset of acute illness, and this condition persisted until death of the animal or until recovery was definite.

Only 12 of 56 (21.4%) monkeys showed respiratory symptoms. Dyspnea was observed, respectively, in five, two, and three animals during the early, acute, and late stages of the infection. Similarly, râles were detected in two, one, and one during these stages. The infrequency and irregularity with which respiratory signs were observed suggest that primary rickettsial pneumonia was not established after aerosol challenge, although serous pneumonitis was observed, microscopically, at autopsy (see below).

Rash, similar to that seen in man, was observed in 38 of 56 (67.9%) rhesus monkeys that became ill, and usually appeared 9 to 13 days

after challenge. Only 13 of 23 (56.5%) animals that died before the 12th day exhibited rash, whereas 18 of 19 (94.7%) succumbing on the 12th day or later showed skin manifestations. In nonfatal infections, 7 of 14 showed skin lesions. Rash was observed most frequently on the extremities and head, as shown in Table 2, but the anatomical area first affected was variable (17).

Peripheral necrosis was observed 13 to 24 days after challenge in 9 of 56 (16.1%) rhesus monkeys (Table 1). The ears were affected in all nine, and two also had necrosis of the fingertips. Six of the nine had recovered and were otherwise normal when necrosis was first observed. The remaining three died 18 to 22 days after exposure.

Mortality in monkeys exposed to aerosols of *R. rickettsii* was dose-dependent to a limited degree only (Table 3). One of four monkeys retaining 1.5 YSLD₅₀ exhibited typical symptoms and rash and died on the 15th PED, whereas the remaining three animals showed no symptoms or laboratory evidence of infection. All of six monkeys retaining 4 to 6 YSLD₅₀ were infected, and five of six died. However, although all of seven monkeys retaining 11 YSLD₅₀ became ill, only three died. In other experiments involving 24 monkeys and retained doses ranging from 67 to 450 YSLD₅₀, there was little or no relationship between retained dose and mortality. This poor correlation between mortality and the number of rickettsiae retained during exposure is shown by the fact that, with varying doses of less than 20, 20 to 200, 200 to 500, and more than 500 YSLD₅₀, 9 of 17 (52.9%), 6 of 10 (60.0%), 13 of 14

TABLE 3. *Relationship between retained dose, morbidity, and mortality in rhesus monkeys after aerosol exposure to Rickettsia rickettsii*

YSLDu retained	Morbidity	Mortality
1.5	1/4[a]	1/4[a]
4	2/2	2/2
5	2/2	2/2
6	2/2	1/2
11	7/7	3/7
67	1/2	1/2
107	2/2	1/2
138	2/2	1/2
156	2/2	2/2
191	2/2	1/2
305	7/7	6/7
333	2/2	2/2
341	2/2	2/2
342	1/1	1/1
450	2/2	2/2
555	2/2	1/2
650	2/2	2/2
1,013	2/2	1/2
2,475	2/2	2/2
2,745	3/3	1/3
3,210	2/2	2/2
3,330	2/2	2/2
7,950	2/2	1/2
9,000	2/2	2/2
Total	56/60	42/60

[a] Number ill/total number exposed.

TABLE 4. *Laboratory findings in 56 rhesus monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Finding	No. of monkeys	Per cent
Positive CRP	56	100.0
Leukocytosis	23	41.1
Left shift in differential	55	98.2
Decrease in hematocrit	40	71.5
Decrease in hemoglobin	48	85.7

[a] Data from Saslaw et al. (17).

TABLE 5. *Isolation of rickettsiae from peripheral blood of monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Days postchallenge	No. of isolations attempted	No. of isolations successful	Per cent
1-5	17	0	0.0
6	1	1	
7	8	5	
8	1	1	
9	5	3	
10	2	2	
11	3	2	
Total, 6-11 days	20	14	70.0
12-18	10	2	20.0

[a] Data from Saslaw et al. (17).

(92.9%), and 14 of 19 (73.7%) animals, respectively, experienced fatal infection.

On the other hand, the incubation period seemed to be inversely related to dose. For example, the means of the days on which monkeys became febrile after receiving less than 20, 20 to 200, 200 to 500, and more than 500 YSLD$_{50}$ were 8.7, 6.4, 6.2, and 5.5 days, respectively. Similarly, the means of the days on which rash was first observed in animals in the four dose groups were 12.6, 11.2, 11.1, and 10.1 days, respectively.

Only eight cynomolgus monkeys were utilized in these studies. Two and six cynomolgus monkeys received 468 and 1,013 YSLD$_{50}$, respectively. All 8 died at 10 to 13 days postchallenge. The acute, rapidly fatal process noted in cynomolgus monkeys was similar to that observed in rhesus monkeys receiving similar doses.

Laboratory Aspects

Laboratory findings in rhesus monkeys exposed to aerosols of *R. rickettsii* included positive C-reactive protein (CRP) reactions, a rather dramatic left shift in neutrophils with or without leukocytosis, anemia, rickettsemia, and the appearance of OX-19 agglutinins and complement-fixing antibodies. CRP usually was first detected on the first day of fever, i.e., 1 to 3 days prior to appearance of other symptoms All of 56 monkeys that became ill exhibited positive CRP reactions (Table 4). CRP tests were positive until death in fatal infections and became negative 1 to 3 days after clinical improvement was noted in survivors. The left shift in the differential count was also apparent 1 to 3 days before onset of illness in all of 56 monkeys, except for 1 that became only mildly ill. Anemia was observed regularly in acutely ill animals 1 to 3 days after appearance of symptoms (Table 4), but normal values were restored about 1 week after clinical improvement.

Rickettsemia was readily demonstrable 6 to 11 days postchallenge. A total of 47 blood samples taken from 10 monkeys at 1 to 18 days after exposure were inoculated into guinea pigs. As shown in Table 5, none of 17 samples obtained 1 to 5 days after challenge yielded rickettsiae, whereas 14 of 20 (70.0%) samples drawn on the 6th through 11th days produced typical symptoms, febrile response, and complement-fixing

Table 6. *Antibody response of monkeys surviving infection after respiratory challenge with Rickettsia rickettsii*[a]

Monkey no.	Complement-fixing antibody titer			OX-19 Agglutination titer		
	Base line	Peak	Day of peak titer[b]	Base line	Peak	Day of peak titer[c]
2	-	320	28	--	160	21
3	--	320	28	--	--	--
4	--	160	28	20	80	21
5	-	640	28	-	--	--
6	--	640	28	-	10	21
7	--	640	14	--	--	--
8	---	320	28	--	160	14
9	--	640	14	5	160	14
10	--	320	14	--	--	--
11	--	80	35	5	160	14
12	--	320	21	5	40	14
13	--	160	28	--	20	14
14	--	320	14	--	20	14
15	--	640	21	--	80	21

[a] Data from Saslaw et al. (17).
[b] Mean, 23.5 days.
[c] Mean, 16.8 days.

antibodies in guinea pigs. Only 2 of 10 samples taken after the 11th day yielded *R. rickettsii*.

Homogenates of tissues taken at autopsy from 13 monkeys with fatal infection were also inoculated into guinea pigs. *R. rickettsii* was isolated, respectively, from spleen, liver, kidney, lung, and brain of 9 of 13, 6 of 10, 6 of 7, 6 of 8, and 5 of 6 animals. Rickettsiae were recovered from heart blood in only one of five monkeys, although they were isolated from other tissues from all five.

All monkeys surviving long enough to allow serial serological studies showed significant rises in complement-fixing antibody titers (Table 6), with peak titers at 14 to 35 days (mean, 23.5 days) postchallenge. In contrast, only 10 of 14 showed significant titer changes in the Weil-Felix agglutination test, with peak titers 14 to 21 days (mean, 16.8 days) after challenge.

Pathology

Gross and microscopic pathology in monkeys dying after exposure to aerosols of *R. rickettsii* were similar to those observed in monkeys infected by other than the respiratory route (11). The lungs consistently showed patchy serous pneumonitis, which was characterized by septal widening associated with serous exudation and swelling and proliferation of capillary endothelial cells. Obvious pneumonia was not observed.

Thus, although the respiratory tract was the portal of entry, the pathological findings were consistent with the clinical observation that pulmonary involvement was not a major factor in the outcome of the disease. Severe lesions of the skin, ears, and nasal septae were characterized by vascular thrombosis and necrosis. Other findings included pericardial effusion, localized myocardial capillaritis, glomerulitis and intertubular capillaritis, endarteritis of the pulmonary vessels, fatty degeneration of the liver, and splenomegaly with lymphoid depletion for the first 2 weeks, followed by regeneration. Congestion of the tunica albuginea was a frequent lesion, as was congestion or hemorrhage, or both, of the adrenal cortex and medulla. No gross or microscopic lesions were seen in the brain.

Immunity Studies

Response of Vaccinated Monkeys

In an attempt to establish the degree and duration of immunity conferred by vaccination, monkeys were challenged with aerosols of *R. rickettsii* at intervals up to 1 year after three weekly subcutaneous injections of 0.25 ml of commercial vaccine (Lederle Laboratories, Pearl River, N. Y.). In the first of four experiments (Table 7), four vaccinated monkeys showed no clinical signs of illness after challenge with 650 $YSLD_{50}$ at 3 weeks postvaccination, whereas two control monkeys exhibited typical symptoms and died on the 9th and 10th PED, respectively. Results of laboratory studies indicated that subclinical infection had been established in three of four vaccinated monkeys. For example, three of four exhibited positive tests for CRP and characteristic changes in the leukocytes; one of four showed a slight but significant fever, and all four showed significant increases in complement-fixing antibody titer after challenge.

Similarly, four monkeys challenged with 341 $YSLD_{50}$ 6 weeks after vaccination exhibited no symptoms after exposure, but laboratory evidence of infection was obtained in two of four, and all four showed significant rises in complement-fixing antibody titer. Both control animals became acutely ill and died 11 and 12 days, respectively, after challenge.

In contrast, when monkeys were challenged 6 months postvaccination with 2,745 $YSLD_{50}$, one of three became ill, and all three showed significant fever, positive CRP reactions, and characteristic changes in the differential count; however, all three survived. All three control animals became acutely ill, and two of three exhibited typical rash. One or three died on the 9th PED, and the remaining two survived. When two monkeys

TABLE 7. *Comparison of response of vaccinated and previously infected monkeys after aerosol challenge with Rickettsia rickettsii*

Response	Weeks after vaccination				Weeks after first challenge			Vaccination controls	Rechallenge controls
	3 (a50)ª	6 (341)	26 (2,745)	52 (1,980)	8 (704)	26 (380-11,745)	52 (1,980)		
Symptoms	0/4ᵇ	0/4	1/3	2/2	0/2	0/13	0/4	10/10	13/13
Fever	1/4	2/4	3/3	2/2	0/2	6/13	4/4	10/10	13/13
Rash	0/4	0/4	0/3	1/2	0/2	0/13	0/4	8/10	10/1ᵈ
Positive CRP	3/4	2/4	3/3	2/2	0/2	11/13	4/4	10/10	13/13
Leukocyte changes	3/4	2/4	3/3	2/2	0/2	8/13	3/4	10/10	13/13
Died	0/4	0/4	0/3	1/2	0/2	0/13	0/4	8/10	10/13
Complement-fixation antibody rise	4/4	4/4	3/3	1/1ᶜ	0/2	12/13	4/4	2/2ᶜ	3/3ᶜ

ª Numbers in parentheses show the YSLD₅₀ retained.
ᵇ Number showing symptoms over total number challenged.
ᶜ Survivors.

were challenged with 1,980 YSLD₅₀ 1 year post-vaccination, both became ill, and one showed extensive rash and died on the 10th day. All three control animals experienced acute illness and died on the 10th or 11th day. The clinical picture in both vaccinated monkeys was almost identical to that seen in control animals.

Response of Monkeys After Rechallenge

Two monkeys that had become ill after aerosol challenge, and that had recovered spontaneously, were rechallenged with 264 YSLD₅₀ 2 months later (Table 7). Neither showed any evidence of infection; both remained asymptomatic, and all laboratory tests were negative. Both control animals became ill and exhibited typical rash; 1 of 2 died on the 21st PED. Thus, the first infection apparently conferred a solid immunity. Both rechallenged monkeys had shown peak complement-fixing antibody titers of 1:320 after first exposure. Titers had dropped to 1:80 in both by the time of rechallenge and did not change after the second exposure.

Thirteen monkeys that had been previously infected and treated successfully with antibiotics (see below) were rechallenged 6 months later with doses ranging from 300 to 11,745 YSLD₅₀. None exhibited clinical signs of illness, whereas all of eight control animals became acutely ill and six of eight died. However, the rechallenged monkeys were not solidly immune, in that fever, positive CRP reactions, and changes in the differential leukocyte count were noted in 6 of 13, 11 of 13 and 8 of 13, respectively, after the second exposure. Complement-fixing antibody titers ranged from 1:10 to 1:80 at the time of rechallenge, and rose significantly in 12 of 13 monkeys to 1:40 to 1:640 after rechallenge.

In addition, four infected monkeys that had recovered after antibiotic therapy were rechallenged 1 year later with 1980 YSLD₅₀. None became ill, but all four exhibited significant fever and positive CRP tests, and three of four showed changes in the differential count. Three additional monkeys that had been vaccinated and challenged by aerosol 6 months later (see above) were challenged a second time in this experiment, i.e., 1 year after vaccination. The combination of vaccination and challenge resulted in solid immunity, in that all three monkeys remained well and all laboratory tests were negative. Three control animals died on the 10th or 11th day.

Thus, it would appear that as in man (29), infection apparently resulted in immunity of longer duration than did vaccination. None of 19 monkeys rechallenged 2, 6, or 12 months after recovery from established infection became ill. No illness was observed in monkeys challenged 6 weeks after vaccination. However, one of three and two of two challenged 6 and 12 months, respectively, after vaccination showed typical symptoms. One monkey exposed 12 months postvaccination exhibited extensive rash and died on the 10th day.

Antibiotic Therapy and Prophylaxis

The response of monkeys infected by the aerosol route to appropriate antibiotic therapy was similar to that observed in man after natural infection. Therapy was not instituted in monkeys until at least 48 hr of significant fever and other signs consistent with well-established diesase were present. Monkeys treated with tetracycline, demethylchlortetracycline, and chloramphenicol became afebrile and asymptomatic within 1.5

TABLE 8. *Results of therapy of Rocky Mountain spotted fever in monkeys[a]*

Expt no.	Challenge dose (yolk sac LD50)	Drug[b]	Dose (mg/kg)	Days	Deaths Treated[c]	Deaths Controls
1	4	DMC	25.0	7	1/4[c]	2/2
2	5	DMC	25.0	7	1/6	2/2
3	156	DMC	25.0	3	0/3	2/2
		DMC	12.5	11		
		DMC	12.5	14	0/3	
4	3,210	DMC	8.5	3	1/3	2/2
		DMC	8.5	5	0/3	
		DMC	8.5	7	0/2	
5	107	TC	14.0	3	0/3	1/2
		TC	14.0	5	0/2	
		TC	14.0	7	0/2	
6	191	DMC	30.0	2	0/5	1/2
			15.0	5		
		TC	50.0	2	0/4	
			25.0	5		
7	2,475	C	75.0	2	0/6	2/2
			50.0	5		
		E	50.0	2	1/4	
			25.0	5		

[a] Data from Saslaw et al. (16).

[b] DMC = demethylchlortetracycline (Declomycin); TC = tetracycline (Achromycin V); C = chloramphenicol (Chloromycetin); E = erythromycin estolate (Ilosone).

[c] Number dead per total number.

to 3.5 days (16). Erythromycin estolate was somewhat less effective, and recovery occurred more slowly from 2 to 6 days after institution of therapy. As can be seen in Table 8, only 4 of 50 treated monkeys died, whereas 12 of 14 untreated controls succumbed. Thus, the effectiveness of broad-spectrum antibiotics in treatment of naturally occurring Rocky Mountain spotted fever was also demonstrable when the respiratory route served as the portal of entry.

Studies on prophylaxis (16) showed that administration of 30 and 50 mg/kg per day of demethylchlortetracycline and tetracycline, respectively, for 3 days prior to challenge merely delayed onset of symptoms for 2 days, and deaths occurred 4 to 5 days later than in control animals. When the same doses of each drug were given daily for 3 or 5 days, beginning with the day of exposure, signs of illness were delayed

for about a week, but six of nine died as compared with one of two in controls.

STUDIES ON PERSISTENCE OF *R. RICKETTSII* IN MONKEYS

Limited studies were conducted to determine whether *R. rickettsii* could be recovered from monkeys that survived infection spontaneously or after antibiotic therapy. In addition, previously vaccinated monkeys that were subsequently challenged were included.

Of 4 monkeys that recovered spontaneously and were sacrificed 25 to 75 days after challenge, *R. rickettsii* was isolated by guinea pig inoculation of splenic tissue from one monkey sacrificed on the 27th day. The spleens and other tissues of the other three failed to yield rickettsiae.

The only other monkey from which *R. rickettsii* was isolated had been treated successfully with demethylchlortetracycline for 7 days and sacrificed on the 48th day. The organism was isolated from a bronchial lymph node, but not from other tissues.

No isolates were obtained from 25 other treated monkeys sacrificed 25 to 75 days after challenge. Similarly, no isolates were obtained from 6 monkeys previously vaccinated and then sacrificed 27 to 98 days after challenge.

Although other avenues of approach may have yielded evidence of persistence of *R. rickettsii* in tissues of infected monkeys, these studies via guinea pig inoculation resulted in isolation in only two instances, 27 and 48 days after challenge. Various studies have demonstrated the presence or recrudescence of rickettsiae long after initial infection (9). Attempts to activate infection with cortisone were not successful in 16 monkeys. It is conceivable that other stresses, such as X irradiation, or the use of tissue culture or immunofluorescence, may have resulted in detection of rickettsiae more frequently.

COMMUNICABILITY

During the entire course of these studies, there was no evidence either clinically or serologically of monkey-to-monkey transmission of disease. Normal monkeys kept in the same or adjacent cage showed no evidence of experience with *R. rickettsii* even when contacts were made shortly after aerosol challenge of test monkeys. This is consistent with observations of the nonappearance, in the absence of arthropod vectors, of naturally occurring infection in man (5) or of naturally occurring infection of guinea pigs, as seen in our laboratory.

DISCUSSION

There are occasions in the practice of medicine when the exact source or portal of entry of the infectious agent cannot be established with certainty. Similarly, in laboratory-acquired infections, there has been increasing evidence that infections have occurred in the absence of laboratory accidents or poor technique (26). This has led to a greater appreciation of the potential role of "aerogenic transmission" of a wide variety of diseases. In recent years, studies of experimental tularemia in monkeys (3) and man (18, 19) have demonstrated the infectivity of aerosols of *Francisella tularensis* and have confirmed the suspicion that tularemia had been acquired in laboratories by the respiratory route (26). These observations support other studies which have suggested that the respiratory tract could serve as a portal of entry even in arthropod-borne infections, such as typhus, rickettsialpox, yellow fever (24), Venezuelan equine encephalomyelitis (10, 20, 21), and St. Louis encephalitis (25).

Spencer and Parker (22), among others, recognized that infection of laboratory workers with *R. rickettsii* was frequently observed in the absence of a history of tick bite. Their studies showed that infection in guinea pigs could be induced by instillation of the organism in the conjunctival sac or by placing a drop of infected tick tissue suspension in the mouth. The respiratory route was not investigated. The results of our studies with *R. rickettsii* aerosols in monkeys suggest that some cases of Rocky Mountain spotted fever in laboratory workers could have been due to inhalation of aerosols. Monkeys were highly susceptible to *R. rickettsii* administered by aerosol; one monkey which retained only 1.5 YSLD$_{50}$ became acutely ill, exhibited typical rash, and died, as did five of six receiving four to six YSLD$_{50}$. Of further importance is the fact that the clinical response in monkeys exposed to aerosols of *R. rickettsii* was similar to that seen in monkeys infected by other routes (1, 27). Thus, accidental aerosol exposure of man might be expected to result in disease indistinguishable from that resulting from a tick bite. Of additional significance is the similarity between clinical and laboratory findings in monkeys and those observed in naturally occurring Rocky Mountain spotted fever in man.

As in man, high fever was a constant finding in monkeys exposed to aerosols of *R. rickettsii*, but morning remissions of fever seen in man (5, 29) were not observed in monkeys. The appearance of fever and symptoms 5 to 7 and 6 to 9 days, respectively, after aerosol exposure

of monkeys is in agreement with the observed incubation period in man (29). Also as in man, monkeys first showed lethargy and weakness, followed by acute illness terminating in prostration and coma. The abnormal heart sounds heard in monkeys during the acute stages were similar to those in man (2).

Monkeys exposed to aerosols of *R. rickettsii* exhibited rash and peripheral necrosis similar to those observed in the naturally occurring infection in man, but the distribution of the rash was different. In monkeys, rash was observed most frequently on the extremities and head. Involvement of the trunk was limited to the lower back, perianal, and perineal regions. In the classic disease in man, rash begins on the wrists and ankles and spreads to the trunk, so that the entire body may be involved. None of the monkeys exposed to aerosols showed typical rash on the chest or abdomen. However, the rash in man may be minimal and fleeting in mild cases (5). As in man, peripheral necrosis in monkeys usually involved only the ears and digits. In addition, necrosis of the tail, tip of the penis, and skin over the patella was noted in one monkey each.

Laboratory findings in monkeys exposed to *R. rickettsii* aerosols were also similar to those in Rocky Mountain spotted fever in man. Anemia was observed regularly in acutely ill animals. A marked left shift of neutrophils was noted in 55 of 56 monkeys that became ill after exposure; about 50% exhibited significant leukocytosis. Although opinions differ as to the frequency with which changes in the leukocytes are observed in man, Harrell (5) has stated that, as the disease progresses, leukocytosis with a left shift in the differential leukocyte count is noted.

Rickettsemia was readily demonstrable, by guinea pig inoculations, 6 to 11 days after exposure, but not during the first 5 days and only infrequently after the 11th day. In man, rickettsiae can be recovered from the blood throughout the 1st week of illness and during the first part of the 2nd week (5) with sufficient regularity to make the guinea pig infection test a reliable diagnostic procedure.

As in man, surviving monkeys showed OX-19 agglutinins and complement-fixing antibodies. All of 14 exhibited significant increases in complement-fixing antibodies, whereas significant titer changes were noted in only 10 of 14 as measured by the Weil-Felix test. Peak OX-19 agglutination titers were observed 14 to 21 days (mean, 16.8 days) after exposure, whereas peak complement-fixing antibody titers were not attained until 14 to 35 days (mean, 23.5 days) postchallenge. In Rocky Mountain spotted fever

in man, OX-19 agglutinins are almost always present by the 12th day of illness (29), although a few patients never show a positive Weil-Felix test. Complement-fixing antibodies appear during the 2nd or 3rd week of illness, i.e., later than OX-19 agglutinins. Hersey, Colvin, and Shepard (8) have shown that the complement-fixation test is more sensitive than the Weil-Felix reaction in detecting Rocky Mountain spotted fever in man.

Studies of the effectiveness of vaccination in monkeys and the response of monkeys to rechallenge provided further similarities in *R. rickettsii* infections in monkeys and man. None of 19 monkeys rechallenged 2, 6, or 12 months after recovery from established infection became ill. Vaccination was effective in preventing symptoms after challenge 6 weeks later, but one of three and two of two challenged 6 and 12 months, respectively, after vaccination showed typical symptoms, and one of two exposed 12 months postvaccination became acutely ill, exhibited typical rash, and died. It is generally agreed that, in man, recovery from infection confers a higher level of immunity of longer duration than does vaccination.

In addition, the response of infected monkeys to antibiotic therapy was similar to that observed in Rocky Mountain spotted fever in humans. Monkeys became afebrile and asymptomatic 1.5 to 3.5 days after institution of therapy with tetracycline, demethylchlortetracycline, or chloramphenicol. Erythromycin estolate was somewhat less effective, however. Chloramphenicol, chlortetracycline, oxytetracycline, and tetracycline have proved to be effective chemotherapeutic agents in human infections, but erythromycin failed to alter either the febrile or toxic course of the illness in two patients (29).

Thus, throughout these studies of monkeys exposed to aerosols of *R. rickettsii*, remarkable similarities to the naturally occurring infection in man were observed. Additionally, the clinical picture in monkeys exposed to aerosols was similar to that observed by others in monkeys challenged by other than the respiratory route. From these results, it would be predicted that exposure of man to *R. rickettsii* aerosols would result in illness much like that observed after a tick bite. These studies in monkeys would suggest, therefore, that aerogenic transmission should be considered in infections of laboratory personnel who have had no known contact with ticks.

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Discussion

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Rocky Mountain spotted fever (RMSF) in man is rarely acquired by means other than tick bite. Nevertheless, a number of instances have occurred in the past, especially among laboratory workers, in which it was possible to infer that some of these rickettsial infections were transmitted aerogenically (3, 4).

Saslaw and Carlisle have presented incontrovertible experimental evidence that the etiological agent of RMSF, in remarkably small doses, can produce a true airborne infection in monkeys. A point to be emphasized is that a disease naturally transmitted by ticks can also be induced by aerosols of the microorganism. During the past decade or more, it has been demonstrated that a number of classical arthropodborne diseases can be established in laboratory animals exposed to aerosols of the respective microorganisms; Venezuelan equine encephalitis (6), Rift Valley fever, and yellow fever are a few examples (2).

A question that arises is: do aerosols of these infectious microorganisms play any role in the ecology of the diseases they produce? In analyzing the question, one comes to th · conclusion that aerogenic transmission has little, if any, influence on the ecological aspects of the arbovirus diseases mentioned. It is tempting to speculate, however, that the aerosol stability of some of the rickettsiae might be of some importance in determining whether they can also be aerogenically transmitted. It can be shown experimentally that the aerosol stability of the rickettsiae of RMSF and typhus is relatively low, considerably lower, in fact, than that of the rickettsiae of Q fever (unpublished data), which is characteristically transmitted by aerosol in man. It is interesting to note parenthetically that RMSF and Q fever resemble each other ecologically in b   : involved with the same biotic tetrad—the ri ...ttsiae, the ticks, vertebrates (mainly mammals), and man. The importance of ticks in the maintenance of both diseases in nature is unquestioned; yet, in man RMSF is caused almost

exclusively by infected ticks, and Q fever is transmitted almost exclusively by aerosols. Thus epidemiological disease patterns may be influenced to some extent by the survival capacity of infectious microorganisms in the airborne state.

Another point of interest concerns the treatment of RMSF with certain antibiotics, which are, without question, highly effective therapeutic agents against the disease. Before the advent of antibiotics, mortality was exceedingly high and RMSF greatly taxed the symptomatic and supportive therapeutic resources available. One can see a dramatic reduction in mortality from approximately 20 to 5% since 1949, the year when antibiotic treatment was first introduced. It is a matter of record also that, with the availability of the highly effective therapeutic agents, the utilization of vaccine has correspondingly decreased—except perhaps for those at great risk. Despite the gradual decrease in the total number of cases reported each year since 1949 in the United States, and the reduction in the case-fatality rate, RMSF continues to be an important disease, especially among children in the South Atlantic States (5). Atwood et al. (1) have presented evidence which indicates that "the true incidence of RMSF is currently much greater than the number of reported cases." One reason for the discrepancies in the statistical data is the wide use of broad-spectrum antibiotics early in the course of many febrile illnesses. It was because of these facts and the almost complete reliance on antibiotics in dealing with this disease that an attempt was made to determine whether the rickettsiae of RMSF could be rendered resistant to selected antibiotics.

Employing the procedures of Weiss and Dressler (7) with some modifications, we exposed rickettsiae of RMSF in continuous passage to erythromycin (73 passages), oxytetracycline (55 passages), and chlortetracycline (50 passages). Application of high antibiotic concentration to a large number of infected eggs was also attempted to isolate resistant mutants by subsequent passage in eggs injected with the same three antibiotics. In limited experiments, ultraviolet radiation was also employed as a mutagen. Under these kinds of experimental conditions, no anti-

biotic-resistant mutants have been isolated. This is not to be interpreted to mean that these rickettsiae cannot become antibiotic resistant. The matter of mutation rate may be involved—one much lower than 10^{-7} or 10^{-9}. The data do suggest, however, that the chance appearance of antibiotic-resistant strains of RMSF is remote.

Although the danger of the rickettsial diseases has diminished, most of these infections remain widespread, constituting an ever-present threat to human health. RMSF will probably continue to be a problem in the United States, affecting about 200 persons or more annually. The number of cases might, in fact, increase because trends in land use seem to be increasing the amount of area suitable for tick habitation, and because many such areas are being suburbanized (1). Thus, in this country, RMSF merits continuing vigilance and research into methods for its treatment, control, and eventual eradication.

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Industrial Inhalation Anthrax

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INTRODUCTION

"Woolsorter's disease," or inhalation anthrax, was a serious problem in Europe in the latter part of the 19th century; at least several hundred cases were reported, with an associated high fatality rate. Governmental inquiries conducted in different countries (9, 10, 15, 17) resulted in a series of effective control regulations, including the establishment in Liverpool of a disinfection station (14), where all "dangerous wool and goat hair" had to be decontaminated before being further processed in England.

In the United States, however, inhalation anthrax has never been a serious problem, although workers have routinely been exposed to naturally occurring aerosols while processing imported wool, goat hair, and hides. Since 1900, there have been fewer than 20 cases of inhalation anthrax reported in the United States (13); approximately half of these occurred among individuals with only fleeting contact with materials known to be contaminated. The only reported epidemic of the disease in this country occurred in 1957 among employees at a goat-hair processing mill in Manchester, N.H. (6, 13). Five individuals developed inhalation anthrax over a 10-week period, with four fatalities. Other cases since 1957 have involved a laboratory employee who was accidentally exposed, a secretary in a goat-hair processing mill who entered a highly contaminated area for only a brief moment, and a 27-year-old man with quiescent Boeck's sarcoidosis whose only known contact was in passing the open receiving door of a tannery on his way home from work (7).

It is not clear why more cases have not occurred in goat-hair and woolen mills and in tanneries, especially among employees working in the dustiest areas where the most concentrated *Bacillus anthracis*-containing aerosols are created. It may be that the dose to which employees are

exposed is below the infecting dose for man, or that employees have developed resistance from chronic exposure. It is conceivable that cases have occurred that were not properly diagnosed. Equally unusual has been the sporadic occurrence of cases in people with no industrial exposure. It may be that these individuals are unusually susceptible, as may have been the case with the individual with Boeck's sarcoidosis. More specific information about inhalation anthrax in man is currently difficult to obtain because almost all workers in the high-risk industries within the United States have been immunized (8).

Significant data related to the pathogenesis of the disease and the dose-response relationships have been reported on the basis of animal studies conducted in both this and other countries (1, 2, 4, 16, 19). However, these studies have involved exposures to pure, concentrated aerosols of *B. anthracis* over relatively short periods of time. There have been no reports on the effect of chronic exposure of animals to aerosols containing *B. anthracis*, either homogeneous aerosols, as in laboratory experiments, or heterogeneous aerosols, as in the natural situation in a factory.

At the Conference on Airborne Infection, Riley discussed his studies on airborne tuberculosis in guinea pigs (11), in which he utilized a holding chamber through which air passed from rooms housing patients with sputum-positive, cavitary tuberculosis. Using this physical arrangement as a model, the authors, with Harold Glassman and Elwood Wolfe of Fort Detrick, developed a protocol to study the clinical course, pathogenesis, and dose-response relationships of experimental animals to a naturally occurring *B. anthracis* aerosol produced in a goat-hair processing mill.

MATERIALS AND METHODS

A 40-foot trailer was outfitted at Fort Detrick (under the direction of Harold Curry) as a com-

LABORATORY TRAILER
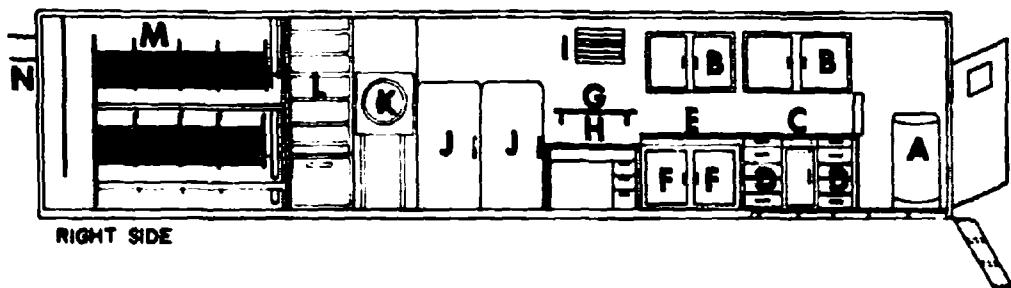
RIGHT SIDE
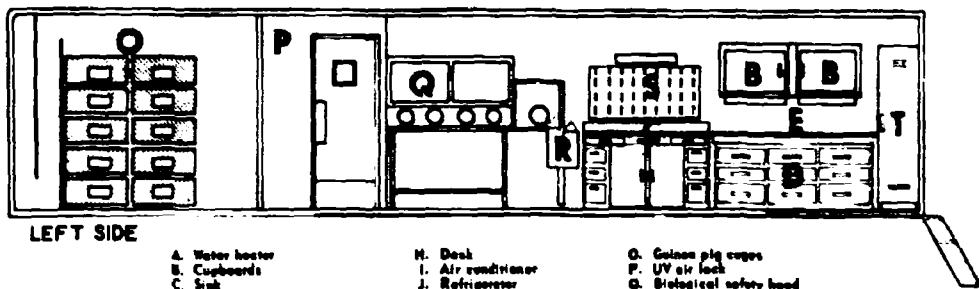
LEFT SIDE

A. Water heater
B. Cupboards
C. Sink
D. Drawers
E. Laboratory benches
F. Incubators
G. Shelf
H. Desk
I. Air conditioner
J. Refrigerator
K. Autoclave
L. Shelves
M. Monkey cages
N. Air intake
O. Guinea pig cages
P. UV air lock
Q. Biological safety hood
R. Hypochlorite dunk bath
S. Equipment drying rack
T. Locker

SCHEME OF ANIMAL EXPOSURE

BUILDING FOR
PROCESSING GOAT HAIR

LABORATORY TRAILER

CONTAMINATED AIR

MONKEY
CAGES

PICKING
MACHINE

BALE OF
GOAT HAIR
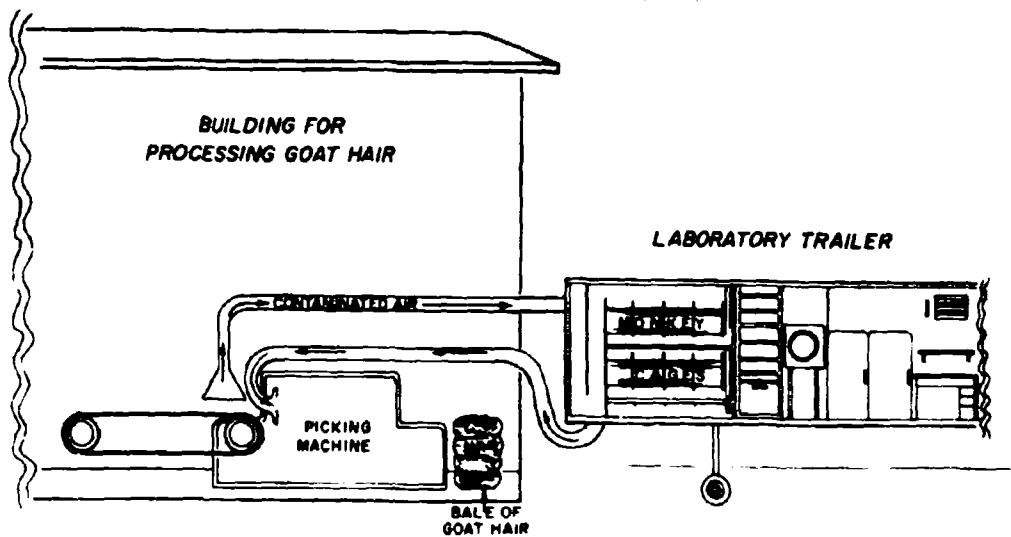
FIG. 1. *Laboratory trailer and scheme of animal exposure.*

bined animal exposure chamber and laboratory and was subsequently located at a mill in South Carolina (Fig. 1). This mill processes goat hair, imported mainly from India and Pakistan, into a woven hair-cloth interlining for suit coats. There were approximately 250 employees, all of whom had been immunized with the Wright anthrax antigen (8). Prior to immunization, 19 cases of cutaneous anthrax had been reported during the 2.5 years the mill had been in operation. Inhalation anthrax had never been reported at this mill.

The aerosol for animal exposure was created around the picking machine, the first machine in the processing cycle, where the clumps of hair were raked apart. The mill usually worked 8 hr a day and 5 days a week, but the picking machine was in operation intermittently during the working day for a total operational time of from 2 to 4 hr. Plastic conduits located in a hood over the picking machine and a suction fan were installed to carry the aerosol from the mill through the

animal exposure chamber and back again to the mill (Fig. 1, bottom). A "T" connection made it possible to bring in outside air when the animals were not being exposed to mill air. The trailer was completely self-sufficient except for water and electricity.

As a result of experiences at Fort Detrick, the cynomolgus monkey was selected as the test animal. Preconditioned 3-lb monkeys imported from Asia were used in all runs. All monkeys were tuberculin-negative; if necessary, they were treated for respiratory disease and parasites but not less than 7 days prior to exposure. The monkeys were grouped two or three to a cage and fed a standard diet, fresh fruit, and water ad libitum. The temperature in the exposure area was controlled between 22 and 33 C. Monkeys were bled for serological studies before they entered the trailer, at intervals during the exposure period, and at the termination of exposure in the case of survivors. Monkeys were observed at least three
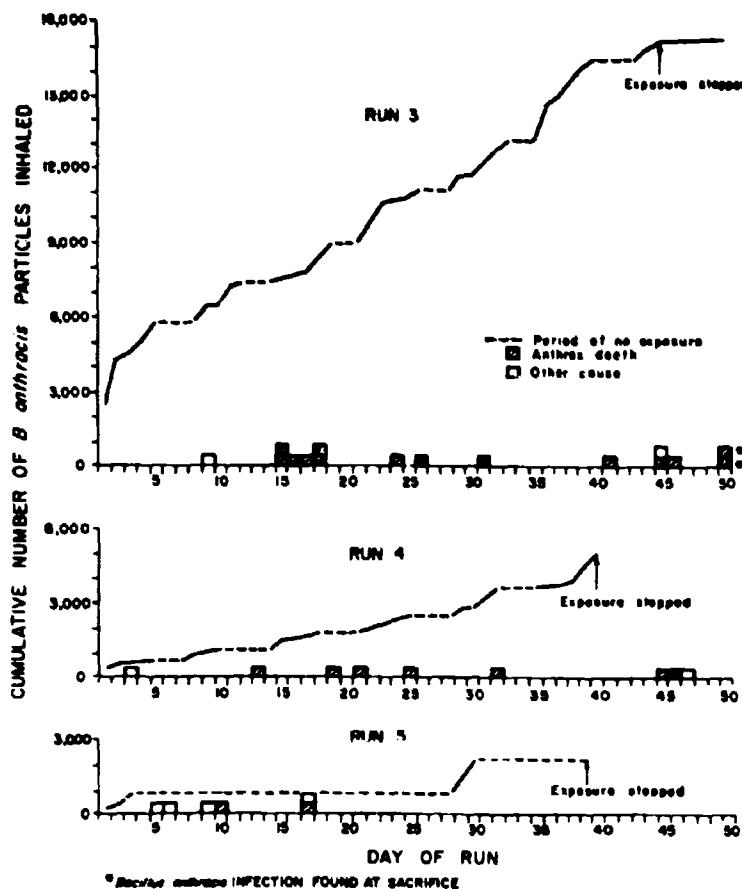

FIG. 2. Occurrence of deaths by day, from beginning of exposure

times a day, and autopsies were performed as soon as possible after death. All surviving animals were sacrificed with an intravenous injection of Nembutal. Postmortem examinations were conducted in general accord with the procedures outlined in the veterinary necropsy protocol of the Armed Forces Institute of Pathology, Washington, D.C. Appropriate cultures were obtained, and the tissue blocks in 10% formaldehyde were returned to Fort Detrick, where Frederic G. Dalldorf, Pathology Division, performed the histological examinations.

With the cooperation of Harry Lefkowitz, Fort Detrick, the protocol for obtaining air samples in the exposure chamber was developed. The all-glass impinger with the British preimpinger was selected as the standard air-sampling equipment to be used (18). Each sampler was run for 20 min, and air samples were obtained throughout all periods during which the monkeys were exposed to mill air. The impinging fluid consisted of 20 ml of gelatin phosphate collecting fluid with 3 drops of a 1:10 dilution of Dow-Corning Antifoam A. The bacterial content of the collected samples was determined by streaking 0.1 ml from the reagitated collecting fluid on each of three 5% human blood-agar plates, which were then incubated at 37 C for 15 to 20 hr. All suspicious colonies were counted, and a representative number were examined by routine bacteriological methods. Calculation of the dose of *B. anthracis*-bearing particles less than 5 μ in diameter inhaled by individual monkeys was based on the various dilution factors, the average number of *B. anthracis* colonies per plate during exposure, and an estimated respiratory rate of 1 liter per min. (All further discussion of the calculated, inhaled dose of *B. anthracis*-bearing
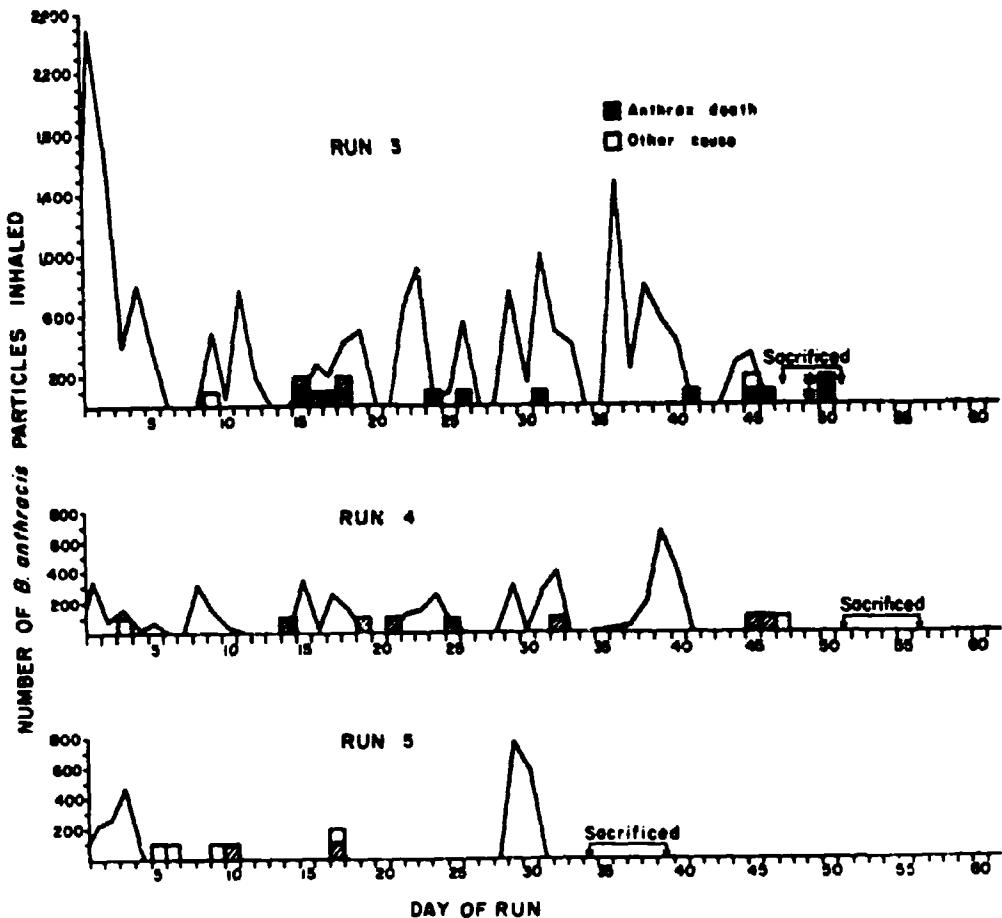
FIG. 3. *Estimated daily dosage (aerosol) per monkey of Bacillus anthracis.*
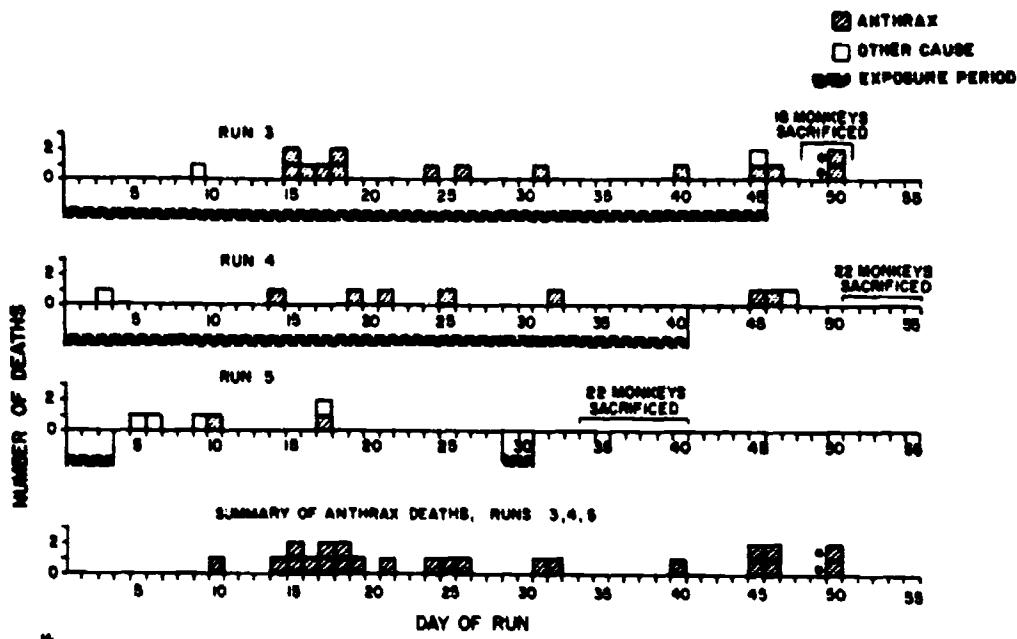
FIG. 4. *Estimated cumulative dosage (aerosol) per monkey of Bacillus anthracis.*

particles refers to those particles less than 5 μ in diameter.)

There were five separate exposure periods extending over 18 months. Because of inadequate preconditioning of the monkeys, difficulties with the environmental control within the exposure chamber, and deficiencies in the collection of air samples and serum specimens, the data from the first two runs were considered to be inadequate for analysis. These technical problems were corrected by changes in the trailer set-up and protocol. The remainder of this discussion will deal with the data developed from the third, fourth, and fifth runs. In the third and fourth runs, the animals were exposed constantly during the working day, regardless of whether the picking machine was in operation, and air samples were collected continuously during this exposure. During the fifth run, exposure was limited to the periods when selected bales of goat hair were actually being picked.

RESULTS

During the third run, 32 monkeys were exposed during 47 consecutive days; 12 of them died of anthrax between the 15th and the 46th day of exposure (Fig. 2, 3, 4, Table 1). Anthrax in 11 of these deaths was diagnosed at necropsy, and, in an additional death, when the microscopic

sections were examined. Two monkeys died of other causes. The 18 survivors were sacrificed and examined; two of them evidenced anthrax infection (no. 51 and 50), one by positive blood culture (no. 51), and both by the presence of organisms resembling *B. anthracis* in mediastinal lymph node sections. Fluorescent-antibody studies of sections from these two monkeys confirmed the identification of anthrax organisms in one (no. 51) and were questionably positive in the other (no. 50). The remaining 16 monkeys appeared to be free from any anthrax infection. The final anthrax fatality rate was 43.8%.

Actual exposure occurred during only 32 of the consecutive 47 days of the third run, and the daily mean for these days was 530 *B. anthracis*-bearing particles. Exposure on the 1st day of the third run was greater than any other single day's exposure in all the runs, being calculated at an inhaled dose of 2,500 *B. anthracis*-bearing particles. A cluster of six deaths occurred between 15 and 18 days after this peak exposure. Other deaths were scattered through the remainder of the run. The total calculated inhaled dose for monkeys surviving the exposure was 16,962 *B. anthracis*-bearing particles less than 5 μ in diameter.

During the fourth run, 31 monkeys were exposed over 41 days. Survivors were held for an additional 10 days before being sacrificed. A

TABLE 1. Summary of the five exposures

Date of exposure	Type of exposure	Length of exposure (animal live days)	Observation period following termination of exposure (days)	Frequency of collection of air samples	Calculated inhaled dose of Bacillus anthracis[a] Total cumulative dose	Avg 24 hr dose	No. of monkeys	Died during exposure Anthrax	Died during exposure Other causes	Survivors Evidence of anthrax infection	Survivors Normal	Total anthrax deaths	Mortality rate (%)
1. 28 January through 28 February 1963	Constant	32	None	Sporadic	[c]	[c]	17	2	13	0	2	2	11.8[d]
2. 25 March through 10 May 1963	Constant days 1-6, intermittent days 7-20, constant days 21-35	47	None	Sporadic	[c]	[c]	20	2	15	0	3	2	10.0[d]
3. 14 October through 29 November 1963	Only when "picking" hair	47	2 to 5	Total	16,962	530	32	12[e]	3	2	16	14	43.8[f]
4. 3 February through 13 March 1964	Only when "picking" hair	41	10 to 15	Total	4,959	198	31	7	2	0	22	7	22.6
5. 1 June through 3 June 1964	Only when "picking" hair	55 hr	25	Total	947	413	28	2	4	[b]	[g]	2	7.1
29 June through 30 June 1965	Only when "picking" hair	31 hr	4 to 8	Total	1,347	1,041	22	0	0	0	22	0	0

[a] Particles less than 5 μ in size.
[b] No evidence of anthrax infection.
[c] Data too fragmentary to use for calculations.
[d] Minimal figure
[e] In one monkey (no. 40), anthrax was considered a contributing cause of death.
[f] Two animals survived and found infected with B. anthracis at autopsy.
[g] Survivors re-exposed.

TABLE 2. *Summary of gross findings (third, fourth, fifth runs—monkeys that died of anthrax)*[a]

Run	Animal no.	Sex	Cumulative days after exposure begun	Mediastinum			Pleural effusion	Parenchymal abnormalities[b]	Adrenal	Spleen	Hemorrhagic meningitis	Culture	Final histopathologic diagnosis
					Nodes	Paratracheal nodes							
3	52	F	15	+	+	−	+	+	+	+	+	+	Anthrax
	66	F	15	−	−	−	−	+	−	−	−	−	Bronchopneumonia and anthrax pneumonia
	40	F	16	+	+	−	−	−	−	−	−	−	Anthrax; staphylococcal septicemia; cachexia
	63	F	17	+	+	−	+	−	+	+	−	+	Anthrax
	43	F	18	+	+	+	+	+	+	+	−	+	Anthrax
	61	M	18	+	+	+	+	−	+	+	−	+	Anthrax
	44	F	24	+	+	+	+	−	+	+	−	+	Anthrax
	69	M	26	−	±	−	+	+	+	−	+	+	Anthrax pneumonia
	55	F	31	+	+	+	+	+	+	+	−	+	Anthrax
	59	M	39	+	+	+	−	+	+	+	−	+	Anthrax
	49	F	45	+	+	+	+	+	+	+	+	+	Anthrax
	60	M	46	+	+	+	−	+	+	+	−	+	Anthrax
	50[d]	F	50	−	−	−	−	−	−	−	−	−	Anthrax in one lymph node
	51[d]	M	53	−	−	−	−	−	−	−	−	+	Anthrax in two paratracheal lymph nodes
4	80	M	14	−	+	−	−	+	−	?	+	+	Anthrax
	110	M	19	+	+	−	+	+	+	+	?	+	Anthrax
	101	M	21	−	+	−	−	+	?	+	−	+	Anthrax
	100	M	25	+	+	−	+	+	?	+	−	+	Anthrax
	87	F	32	−	+	−	−	+	−	+	−	+	Anthrax
	86	F	45	+	+	−	−	−	+	+	?	+	Anthrax
	92	M	46	−	+	+	−	−	?	+	−	+	Anthrax
5	147	F	10	−	−	−	−	−	?	+	−	+	Anthrax
	151	F	17	+	+	−	+	+	+	+	−	+	Anthrax

*Symbols: + = grossly pathologic; − = no significant abnormal findings; ? = questionable abnormal findings.

[b] Hemorrhagic appearance to lungs.

[c] Heavy overgrowth with *Proteus* species.

[d] Sacrificed as normal at end of run.

total of seven monkeys (22.6%) died of anthrax, five during the 41 days of exposure between the 14th and the 46th day, and two during the 10-day postexposure holding period 4 and 5 days after exposure was terminated. Two monkeys died of other causes. None of the sacrificed animals had evidence of anthrax infection. There was actual exposure on 25 days of the total of 41 consecutive days, and the daily mean for these days was 198 *B. anthracis*-bearing particles. The total calculated inhaled dose over the 41-day period was 4,959 *B. anthracis*-bearing particles.

To develop additional specific dose-response data, in the fifth run animals were exposed to as concentrated an aerosol as possible and then held for a prolonged observation period. Accordingly, arrangements were made with the mill to process a maximal number of bales of goat hair through the picking machine as rapidly as possible. Twenty-eight monkeys were initially exposed during three separate periods over three successive days to a calculated aerosol of 947 *B. anthracis*-bearing particles. The animals were then held for an additional 25 days without further exposure to mill air. Two animals died of inhalation anthrax 10 and 17 days after the first day of exposure, and four died of other causes. The limits of the incubation periods for these

TABLE 3. *Summary of microscopic findings—(monkeys that died—third, fourth, and fifth runs)*

Run	Animal no.	Diagnosis	Anthrax bacteremia	Mediastinal lymph nodes[a]	Cervical lymph nodes[a]	Liver necrosis	Splenic necrosis[b]	Anthrax meningitis	Adrenal hemorrhage and necrosis	Anthrax lobar pneumonia	Pulmonary edema[c]	Colonies of bacilli in submucosa of trachea
3	52	Anthrax	+	+++	++	-	+++	+	0	-		-
	66	Bronchopneumonia and anthrax pneumonia	+	++	-	-	+++	-	-	+		-
	40	Anthrax; staphylococcal septicemia; cachexia	-	+	-	-	++	-	-	+		-
	63	Anthrax	+	++	+	+	++	+	+	-		-
	43	Anthrax	+	+++	++	+	+++	+	-	-		-
	61	Anthrax	+	+++	+	-	+	-	0	-		-
	44	Anthrax	+	+++	0	+	+++	-	+	-		-
	69	Anthrax pneumonia	+	+++	-	+	+++	+	+	+		+
	55	Anthrax	+	+++	0	-	+++	-	-	-		-
	59	Anthrax	+	+++	+	+	+++	+	+	-		-
	49	Anthrax	+	+++	+++	+	++	-	+	-		-
	60	Anthrax	+	++	++	-	++	-	+	-		-
	50	Anthrax in one lymph node	-	++	-	-	+	0	-	-	-	-
	51	Anthrax with two paratracheal lymph nodes	+	++	-	-	-	0	-	-	-	-
4	80	Anthrax	+	+++	+	-	++	+	-	-	+	-
	110	Anthrax	+	+++	+	-	+++	+	+	-	+	-
	101	Anthrax pneumonia	+	+++	+	-	+++	-	-	+	++	-
	100	Anthrax	+	+++	+	-	+++	+	-	-	+	-
	87	Anthrax	+	+++	+	-	+++	+	-	-	-	+
	86	Anthrax	+	+++	+	-	++	-	+	-	+	-
	92	Anthrax	+	+++	+	-	+++	-	-	-	-	-
5	147	Anthrax	+	++	+	-	++	-	-	-	-	-
	141	Anthrax	+	+++	+	+	+++	-	-	-	-	-

[a] Key for lymph node morphology: + = anthrax bacilli with follicular necrosis; ++ = bacilli with necrosis and edema; +++ = bacilli with necrosis, edema, and hemorrhage.

[b] Key for splenic morphology: + = sinusoids engorged with neutrophils; ++ = neutrophils in sinusoids plus central necrosis of malpighian bodies; +++ = necrosis of red and white pulp.

[c] Key for pulmonary edema: + = minimal edema; ++ = moderate edema; +++ = marked edema; — = no edema in alveoli. (0 = no tissue submitted.)

two deaths were 7 and 17 days. The fatality rate was 7.2%. Subsequently, the remaining 22 monkeys were exposed during two separate periods over 31 hr to a calculated aerosol of 1,347 *B. anthracis*-bearing particles. No deaths occurred during the following 4-day observation period. The surviving monkeys were sacrificed over a 6-day period, and none revealed evidence of anthrax infection. During the fifth run, 47 guinea pigs were exposed to the same aerosols and held for the same period as the monkeys. None of them died of anthrax.

Dalldorf studied sections from the 91 monkeys on which autopsies had been done. All had lesions attributable to other causes, such as parasites. Twenty-three showed evidence of anthrax infection (Table 2). Twenty died of inhalation anthrax, and in one anthrax was considered a co-primary

cause of death along with staphylococcal septicemia and cachexia due to enteritis. Two monkeys sacrificed at the end of the third run had early infection in the mediastinal lymph nodes only. Nonanthrax deaths were primarily due to pneumonia and enteritis.

The most consistent pathological findings in anthrax-positive monkeys were mediastinal edema, pleural effusion, enlarged hemorrhagic mediastinal lymph nodes, and enlarged, soft spleens. In four instances, gross hemorrhagic meningitis was observed. *B. anthracis* was recovered on culture from all but three infected animals: a sacrificed animal with an early infection, the monkey in which anthrax was considered a contributory cause of death, and a monkey from which the plates prepared at autopsy were heavily overgrown with *Proteus* sp. There was no gross evidence of primary cutaneous or gastrointestinal anthrax, and there were no lesions associated with the oral cavity, including the tonsils.

Histological examination of the tissues showed that infection was largely limited to the reticuloendothelial system, though there was always widespread dissemination of the bacilli through the vascular system at the time of death (Table 3). Tissue response was primarily that of edema, hemorrhage, and necrosis. The mediastinal lymph nodes were infected in all cases, and in a few monkeys the paratracheal lymph nodes were also infected. No primary lesions were found in the trachea or bronchi. In three monkeys, there was evidence of anthrax pneumonia, but this was not considered primary.

Pathological changes noted in other organs as a result of anthrax infection included splenic and hepatic necrosis, adrenal hemorrhage and necrosis, ovarian hemorrhage, and meningitis. The renal glomeruli contained many bacilli, but the kidneys were otherwise normal.

Serological studies were conducted by George Wright, Immunology Branch, Fort Detrick, by use of a micromodification of the Ouchterlony double-diffusion technique. A total of 210 sera, 66 collected before exposure and 144 collected during or after exposure, were tested without demonstration of any antibody titers.

Discussion

The overall fatality rate of 25.3%, indicates the susceptibility of the cynomolgus monkey to naturally occurring, industrially produced aerosols containing *Bacillus anthracis* and attests to the feasibility of the experimental design. The objectives initially outlined have been partially attained. The clinical and pathological effects of

chronic exposure are not dissimilar to those seen after acute exposure in laboratory experiments.

The pathogenesis of inhalation anthrax after chronic exposure is similar to that postulated after acute exposure of laboratory animals or of man naturally exposed, such as was seen in the Manchester, N.H., epidemic. The necropsy findings of mediastinal edema, mediastinal hemorrhagic lymphadenitis and necrosis, and pleural effusion, without tracheal or bronchial lesions and without primary anthrax pneumonia, support the concept that inhaled *B. anthracis* spores are carried to the mediastinal lymph nodes, where they germinate and produce toxin with development of toxemia and bacteremia. Additional evidence to support this concept is found in the necropsy data from the two sacrificed monkeys in the third run; tissues from these monkeys revealed *B. anthracis*-like organisms primarily in mediastinal lymph nodes These animals were undoubtedly in the early stages of disease and presumably would have developed systemic disease and died, had the experiment not been terminated. Histological examination of the necropsy tissue from all monkeys that died of anthrax shows widespread dissemination of *B. anthracis* organisms.

The serological studies do not support the development of subclinical infection. Norman et al. (12) studied sera from 72 unvaccinated employees of a goat-hair mill and found 11 who had anthrax antibodies demonstrated by a precipitation inhibition test. Most of the positive reactions occurred among employees who worked in the dustiest part of the mill.

In discussing industrial anthrax, Brachman and Fekety (5) compared the length of employment in goat-hair processing mills of a group of employees who did not have a history of anthrax infection with the length of employment of employees with a history of previous anthrax infection; they found that the two curves were essentially parallel. This suggests that the length of employment did not influence the development of anthrax. They noted that some cases of cutaneous anthrax occurred in employees who had worked in these mills for 15 to 20 years. Their conclusion: "These workers do not develop subclinical infection or immunity to anthrax by prolonged exposure to the organism."

The studies with monkey sera may support these data; however, it is possible that the serological test employed was not sensitive enough to demonstrate antibodies actually present, that the antigen used was not specific for protective antibodies, or that the inhaled dose was too low to stimulate production of demonstrable antibodies.

Data from the fifth run indicate that, with a total exposure to 947 *B. anthracis*-bearing particles intermittently over a 55-hr period, there were two deaths at 10 and 17 days after first exposure, for a fatality rate of 7.2%. The shortest incubation period possible was 7 days, and the longest was 17 days. The second exposure during the fifth run resulted in an inhaled dose of 1,347 *B. anthracis*-bearing particles over 31 hr. The fact that there were no deaths during the 4-day holding period and the 6-day period during which survivors were sacrificed may represent either a lack of susceptible monkeys or, more likely, an inadequate observation period.

Data from the third and fourth runs are more difficult to interpret, because of the irregular, sawtooth pattern of exposures on successive days, without knowing the effect of repetitive exposures on the monkeys. For example, frequent small doses may stimulate an antibody response that increases an animal's resistance to clinical disease, or repeated exposures may build up a level of *B. anthracis* organisms in the body which causes disease when a certain threshold level is reached. Another possibility is that repetitive exposure over a period of several days increases the chances of the animal's acquiring an infecting dose.

As already discussed, the serological studies did not demonstrate the presence of circulating antibodies, which supports the concept that chronic exposure does not lead to development of resistance to infection. The lack of deaths following the second exposure in the fifth run cannot be assumed to represent protection resulting from the first exposure because of the lack of an adequate observation period after the second exposure.

The peak exposure during the first 5 days of the third run, 5,685 *B. anthracis* particles, may be causally related to the six anthrax deaths that occurred from 15 to 18 days after the first day of exposure. If related, the fatality rate was 20%, (6 of 30), and the incubation period would then have ranged from 10 to 17 days, which is similar to the incubation period in the fifth run.

Another peak exposure occurred from the 36th to the 40th day, when the surviving 20 monkeys were exposed to 3,525 *B. anthracis*-bearing particles with two deaths occurring from 5 to 11 days after exposure. The two animals found to be infected at autopsy may have become infected as a result of contact with this same aerosol, the incubation period would have been from 10 to 14 days for these two monkeys. If all four deaths are related to the last peak aerosol, the fatality rate would be 20%, (4 of 20). It is most likely
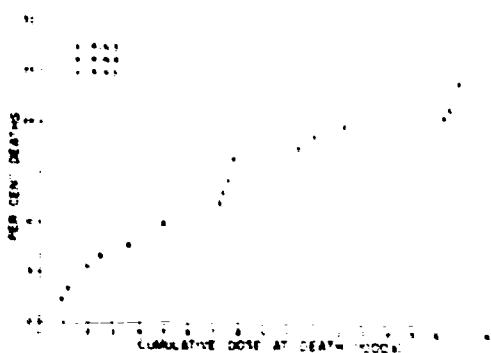
FIG. 5. *Calculated cumulative dose of Bacillus anthracis at time of death.*

that the death on the 41st day was from exposure to the preceding week's aerosol, that is, from days 28 to 33, which would then indicate an incubation period for this particular death of from 8 to 12 days.

The two deaths on successive days at the end of the 4th run (days 45 and 46) occurred 5 to 8 days after a peak exposure to 1,250 *B. anthracis*-bearing particles. If this relationship is correct, then this exposure would be associated with a fatality rate of 8%. The other deaths in the third run and all deaths in the fourth run are harder to associate with specific periods of exposure.

If repeated low-dose exposure results in accumulation of *B. anthracis* organisms until a certain level is reached, after which disease develops, the comparison of the percentage of deaths and the accumulative dose at death should show a straight-line relationship until the critical level is reached, after which there should be a sharp upsurge in the percentage of deaths. As shown in Fig. 5, this is not the case. Additionally, the data were examined to see whether the dose accumulated during specific periods preceding death, that is 7, 10, 12, or 15 days, would suggest an effect of accumulation of *B. anthracis* organisms. Such an analysis is presented in Fig. 6, for which the particles inhaled during the 7 days preceding death are plotted against the day of death of each monkey. Again, the scattering of deaths over a wide range of calculated inhaled doses would tend to be against the theory of accumulation.

Analysis of these data does suggest a dose-response relationship with exposure to approximately 1,000 *B. anthracis*-bearing particles over a 3 to 5-day period, resulting in a fatality rate of approximately 10%. When the exposure is from 3,500 to 5,500 *B. anthracis*-bearing particles over a 5 day period, the rate is from 20 to 25%.

The prolonged incubation periods are un
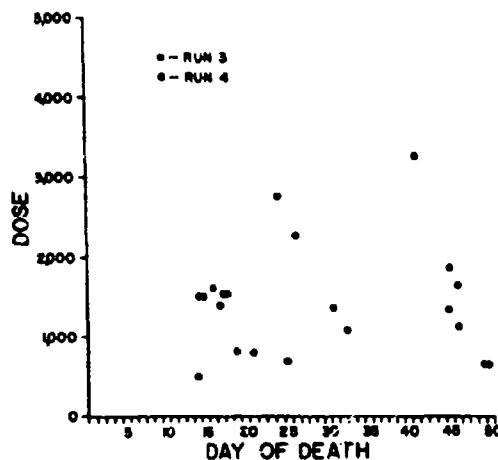
FIG. 6. *Calculated inhaled dose during 7 days preceding death.*

doubtedly related to the low-dose exposure to *B. anthracis.* Exposure to more concentrated, pure aerosols is usually associated with incubation periods of from 3 to 7 days.

Extrapolation of these data to man is difficult. One reason is that man samples a greater proportion of the contaminated mill air because his minute volume is 10 times that of the monkey. There is no reason to suspect any change in the type of goat hair processed or in the method of production from the years before immunization to the present. Thus, the aerosol produced during the three experimental runs is probably representative of the working situation in the picking area at the time when employees were not protected by the anthrax-protective antigen. The lack of cases of inhalation anthrax may therefore represent the lack of exposure to "peak" aerosols as defined above. Also, the monkeys were exposed to a maximal concentration of *B. anthracis* aerosol produced by the picking machine; people, however, are never exposed to the total aerosol produced, but only to a relatively small part of it while they work in the vicinity of the picking machine.

The 1957 epidemic of inhalation anthrax in Manchester, N.H., can possibly be explained by the exposure of the five susceptible individuals to a "peak" aerosol related to a specific batch of hair. In addition, the six sporadic cases that have been reported unassociated with the goat-hair processing industry also may represent the chance exposure of susceptible individuals to a "peak" aerosol.

SUMMARY

Exposure of 91 cynomolgus monkeys to naturally produced aerosols containing *B. anthracis* resulted in an anthrax fatality rate of 25.3%. The pathological findings of mediastinal edema and hemorrhagic lymphadenitis and necrosis are similar to findings in animals after acute exposure to pure aerosols of *B. anthracis*, and also to the findings in humans who have developed fatal inhalation anthrax after industrial or sporadic exposure. With the low-dose chronic exposure to natural aerosols, the incubation period appears to range from 5 to 17 days.

Analysis of the data suggests a dose-response relationship with fatality rates ranging from 10 to 25% after exposure to from 1,000 to 5,500 organisms over 3 to 5 days. There is no specific evidence to support the development of subclinical infection, or of an accumulative effect of anthrax organisms. These studies do support previous concepts concerning the pathogenesis and dose-response relationships of anthrax in monkeys.

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Discussion

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Industrially acquired anthrax has been associated with transportation and processing of imported wool, hides, and goat hair. The spores of *Bacillus anthracis* have been cultivated from as many as 50% of samples of the raw products (4, 15, 17). Industrial processing creates dust, so that anthrax spores regularly contaminate the surfaces and air of the factories; up to 66% of surface samples were positive in three mills processing goat hair (5). Respiratory exposure of workers may reach 510 spores in particles 5 μ or less in diameter in a working period (9). A significant percentage of anterior nasal swabs and pharyngeal washings from mill workers processing goat hair yielded *B. anthracis* (8).

Despite this, inhalation anthrax is rare in the United States (7). Brachman et al. (6) studied the response of the cynomolgus monkey exposed chronically to the air from the dustiest portion of a goat-hair processing mill in an effort to enlarge our understanding of industrial anthrax. It is my purpose to assist in this objective by relating their observations to selected laboratory

studies. Specifically, I will consider the variable incubation period they observed, and the dose-response relationship.

Modern views of the pathogenesis of inhalation anthrax are based on the studies in laboratory animals of the experimentally induced disease by Young et al. (19), Barnes (3), Ross (16), Albrink and Goodlow (2), and Gleiser et al. (10), and the pathological findings in three fatal cases in man reported by Albrink et al. (1). The observations of these investigators agree in defining the role of the lung as a portal of entry in inhalation anthrax; primary anthrax lesions are not found in the trachea or bronchi, at least not in the absence of pre-existing lung lesions. Thus, we may visualize spore-bearing, airborne particulate matter of sufficiently small size (i.e., 5 μ in diameter, or less) after inhalation, penetrating to the deep recesses of the lungs and being deposited there as essentially inert particles. Subsequent removal of the spores is accomplished principally by alveolar macrophages that transport them via the lymphatics to the regional
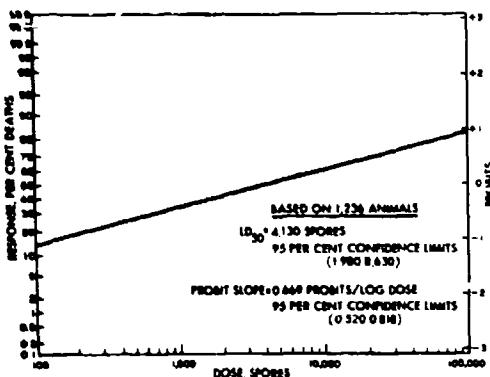
Fig. 1. *Response of the cynomolgus monkey to aerosols of Bacillus anthracis.*

lymph nodes. Neglecting, for our present discussion, the further steps in the pathogenesis of inhalation anthrax, it seems reasonable that a significant period of time will be required for clearance of a large number of spores, even if these are introduced in acute experimental exposures. The respiratory retention of inhaled spores was initially studied by Harper and Morton (12), using radioactively labeled *Bacillus subtilis.* When these were presented to guinea pigs for inhalation as particles 1 μ in diameter, the majority of the radioactivity was found in the lungs (as contrasted with the head or the trachea), and essentially no loss of radioactivity of the lungs was measurable over a 24-hr observation period. Subsequently, in studies of the prophylaxis of inhalation anthrax in the rhesus monkey, Henderson et al. (13) demonstrated that a daily regimen of procaine penicillin, initiated 24 hr subsequent to aerosol exposure, could delay the onset of disease and death, and that this protection ceased promptly on termination of the therapy. They also showed that spores of *B. anthracis* can be detected for as long as 100 days after their deposition onto the lung epithelium. In a more recent similar study, Gochenour et al. (11) provided further direct evidence of prolonged spore retention in the lungs after an acute inhalatory exposure. One of their monkeys died with anthrax meningitis 25 days after completion of an apparently successful course of therapy. Cultures of the lungs of all animals surviving 55 to 84 days after exposure to aerosols of anthrax spores were positive for this organism. Finally, one of my colleagues, Joseph V. Jemski, has made available information he obtained several years ago in making a study of the time to death of the cynomolgus monkey after inhalation of aerosolized anthrax spores. Several of the animals in

that study, which was directed toward determining the minimal holding period required to assure statistically valid dose-response data, died of culturally proven anthrax after prolonged incubation periods—one animal succumbed 98 days subsequent to exposure.

The concepts involved in the pathogenesis of inhalation anthrax, as well as the experimental evidence cited above regarding the prolonged retention of spores in the lungs, are completely consonant with the variable incubation periods reported by Brachman et al. (6) in their studies of chronic exposure to varying doses over many days. An additional pertinent laboratory observation has been the dose dependency of the incubation period, with lower inhaled doses of spores resulting in longer incubation times (10).

In considering the dose-response relationship of the cynomolgus monkey in experimentally induced inhalation anthrax, I am again indebted to Dr. Jemski for placing at my disposal hitherto unpublished data. These represent a compilation of the results of several individual experiments in which large numbers of cynomolgus monkeys were acutely exposed (1 to 10 min) to heterogeneously sized aerosols of anthrax spores. The aerosol clouds were sampled with an impinger preceded by a preimpinger, the latter device screening out the majority of particles greater than 5 μ in diameter (14, 18). Thus, the dose reported, after microbiological assay of the collection fluid of the impinger, represents spores present in particles predominantly 5 μ in diameter, or less. This dose, and the mortality of the monkeys from culturally proven anthrax during a 10-day observation period subsequent to aerosol exposure, have been subjected to statistical analysis by the probit method (Fig. 1).

It will be noted that the median lethal dose (LD_{50}) based upon a total of 1,236 animals is 4,130 spores with 95% confidence limits of 1,980 to 8,630 spores. The probit slope is 0.669 probits per log dose, with 95% confidence limits of 0.520 to 0.818. As a consequence of this unusually low probit slope, large changes in the dose of inhaled spores will result in comparatively small changes in the per cent mortality. For example, a 100-fold range of dose (10-fold above and 10-fold below the calculated LD_{50}) will only change the predicted mortality from 25 to 75%.

These laboratory studies of Jemski et al. have many similarities to the experimental epidemiological investigations of Brachman et al. (6). There are, however, several important differences. The former involved a very large number of animals, acutely exposed to laboratory-grown spores, under well-controlled conditions for

experimental airborne infection. The latter represented the chronic exposure, over many days, of a small group of animals to an uncontrolled, industrially generated aerosol of spores that were present as a result of the industrial use of contaminated animal products. Nevertheless, the dose-response relationship derived from the laboratory data was predictive of the field results, if the cumulative dose of spores inhaled by the monkeys is considered the most important factor in the chronic exposures [see particularly Fig. 4 and 5 of Brachman et al. (6)].

Precise comparisons of the laboratory observations and the field studies are not possible for reasons already mentioned. For the same reasons, conclusions from consideration of both sets of investigations must be drawn with caution. With this caveat firmly in mind, it does seem that the following statements are justified. (i) The dose-response relationships determined with the cynomolgus monkey in the laboratory permitted prediction of the outcome when the same species was exposed chronically to a contaminated industrial atmosphere. (ii) The adequacy of the cynomolgus monkey as a model for predicting the quantitative aspects of the response of man in the industrial environment is open to reasonable doubt.

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Epidemiology of Airborne Staphylococcal Infection

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INTRODUCTION

It is a characteristic of the airborne route of infection, in contrast to transfer by food or water, that whenever there is the possibility of aerial transfer there is almost always also the possibility of transfer by other routes. This is perhaps especially true of the forms of staphylococcal infection that have been most extensively studied, namely, those occurring in hospitals. But, during the last few years, there has been a great volume of work based on the assumption that airborne spread is an important route in the spread of the human staphylococcal disease, and there is therefore a considerable body of information for review.

It is logical and convenient to discuss first the studies on dispersal of staphylococci into the air and, second, the survival of the cocci in, and their carriage by, the air. These aspects can be presented in some precise and quantitative detail. When we come to consider acquisition, we enter an area in which extrapolation and analogy loom large, but sufficient quantitative data have now been accumulated to give some factual foundation to the discussion. Nevertheless, the final summing up as to the probable importance of airborne transfer in relation to other modes of spread is of necessity a product of judgment rather than arithmetic.

Material Reviewed

For the most part this paper is based on a selective review of published reports, with special reference to those on a series of investigations (44, 45, 58, 59) carried out with R.A. Shooter at St. Bartholomew's Hospital, London, England (referred to as S.B.H.).

Use has also been made of a recent study of my own at St. Mary's Hospital, London, England (S.M.H.), a report of which is in preparation. In this study, we sampled the air in two surgical wards, one having a total of 14 or 15 patients in four rooms and the other having 22 beds in an open ward. Petri dishes [diameter, 6 inches (15 cm)] of serum-agar containing phenol-phthalein phosphate (2) were exposed in the rooms for 12 hr (or in part of the experiment for 24 hr) on each of 5 days each week. Nasal cultures were examined from each patient weekly.

The total number of staphylococcus-carrying particles on the air plates was recognized by the phosphatase reaction and either all or, when the numbers of colonies on the plate were larger than three to five, a portion were subcultured and tested for coagulase and for phage type. It was thus possible to make some estimate of the number of coagulase-positive staphylococci settling from the air, and of the proportion with various phage patterns, for attempted correlation with

the strains isolated from the patients' nasal cultures.

For airborne particles with a diameter equivalent to those found to carry *Staphylococcus aureus*, that is about 14 μ (38), the settling rate in colonies per square foot per minute is numerically approximately equal to the volume count expressed as colonies per cubic foot. A 6-inch petri dish has an area of approximately 0.2 ft²; in round figures, therefore, the count on such a plate exposed for 24 hr is about 60% of the number of particles inhaled by an adult person in the same time, since the volume inhaled is ordinarily about 0.3 ft³ per min.

DISPERSAL OF STAPHYLOCOCCI INTO THE AIR

The frequency with which normal individuals harbor *S. aureus* in the nose and on the skin is now well known (56), and such normal carriers are one important source from which the cocci are dispersed. The other source from which staphylococci may be dispersed, especially in hospitals, comprises patients with infected lesions—of skin, wounds, respiratory tract, or gut.

Nose and Skin Carriers

Hare and his colleagues were among the first to define the frequency with which nasal carrier of *S. aureus* liberate the cocci into the environment; they counted the numbers shed into the air of a very small cubicle during exercise. Hare and Ridley (21) found that all but 6 of 19 carriers liberated staphylococci, and 7 gave substantial numbers; this and subsequent work (41) pointed to the special importance as dispersers of persons who harbor staphylococci on the perineum. On the other hand, White (54, 55) emphasized the relation between dispersal of staphylococci and the total numbers present in the nose and on the skin.

One feature that also emerged from these and other studies was the wide individual variation in the number of staphylococci shed into the air by carriers. The individuals at the upper end of the distribution seemed to differ sufficiently from those at the lower end to justify the use of the term "heavy disperser" for them, and the suggestion that such heavy dispersers might be responsible for epidemics of hospital infection stimulated further study of the mechanism of dispersal.

Mechanism of Dispersal

Hare and his colleagues showed that very few staphylococci are liberated into the air directly from the nose of carriers during ordinary activity;

Hare (19) described the liberation by other routes as "outflow" and emphasized the importance of friction with the skin. White (54) had found that the extent to which patients contaminated their bedding was correlated with the numbers of staphylococci found in their nasal cultures. Subsequently, Davies and Noble (14) demonstrated that large numbers of skin fragments are dispersed into the air during the activities known to liberate bacteria; they suggested that most of the staphylococci are carried on such fragments, and they were able to cultivate *S. aureus* from epithelial squames liberated by a known carrier (15).

It thus seemed that the differences among individual carriers in the number of staphylococci that they disperse might be related to (i) the number of cocci on the skin, (ii) the particular area of skin colonized, or (iii) the rate of desquamation. By parallel sampling of air for skin squames and staphylococci, Noble and Davies (37) showed that the last of these was not likely to be the explanation; they thought that the extent of skin carriage was probably the most important determinant. Hare and Ridley (21) had previously suggested that carriage on the skin of the perineum was particularly likely to lead to dissemination, and Solberg (49) not only confirmed this but also showed that, in the absence of perineal carriage, there is a correlation of numbers of staphylococci disseminated with the number found in the nose or on the skin (in his experiments, of the fingers and hand). The importance of the perineal skin as a source for dispersal receives indirect support from observation that, in operating-room clothes, the greatest liberation of skin bacteria seems to be from below the waist and especially through open trouser ends (5, 8). It may be noted that most observers have measured air contamination while the subjects were exercising in some form of cubicle and generally making quite vigorous leg movements; this may perhaps over-emphasize the contribution of the perineum to aerial dispersal.

Frequency and Magnitude of Dispersal

It was clear from the early work of Hare and Ridley (21) that many nasal carriers shed staphylococci into the air while exercising. This has been amply confirmed. On the basis of experiments in small cubicles, Bethune et al. (5) reported that 14 of 38 nasal carriers (from a group of 150 normal people) generated an air contamination level of one *S. aureus* particle per ft³ or more, corresponding to a total liberation of about 100 particles or more in 2 min of walking-on-the-spot. Noble and Davies (37) examined 127 persons, 54 of whom were normal adults whereas the rest

were hospital patients, many with skin disease. The subjects removed all their clothing and then dressed again in a 100 ft² cubicle from which the air could be sampled. Of the whole group, 30, including only 2 of the 54 normal adults, liberated *S. aureus* to 1% of the total flora in the cubicle; this corresponded to the liberation of about 25 staphylococcus-carrying particles or more. Eight liberated more than 10,000 *S. aureus* particles.

More precise estimates of the numbers of staphylococci liberated by carriers have been provided by Solberg (49; *personal communication*), who estimated the aerial contamination resulting from a standardized agitation of a group of persistent carriers' bedding in a special chamber. Solberg found the air counts of staphylococci dispersed during the making of the beds of his carriers to be distributed in a log-normal fashion, and at least 20% of the 126 carriers (drawn from 2,014 patients surveyed) dispersed more than 10,000 staphylococcus-carrying particles in the standard test (Fig. 1).

Our own studies of air counts in a hospital ward offer another basis for estimating the frequency of dispersal. In Fig. 2 are plotted the mean daily counts of *S. aureus* of the phage type carried by each of the patients who were carriers on admission to the subdivided S.M.H. ward,

FIG. 2. *Air counts (particles per square foot settling in 24 hr) of Staphylococcus aureus generated by patients admitted as carriers to a hospital ward (S. M. H.).*

FIG. 1. *Air counts of Staphylococcus aureus. (A) Generated by disturbance of bedding of persistent carriers [after Solberg (49), supplemented by a personal communication]; (B) during undressing and redressing [after Noble and Davies (37)]. Plotted on a probability scale, so that a straight line represents a normal distribution.*

excluding those who were carrying strains of types already known to be present in the ward; some of the patients, in contrast to Solberg's subjects, were only transient carriers. The counts are clearly also distributed log-normally. About 50% of carriers generated air counts below 5 colonies per ft² per 24 hr, but 10% generated counts that averaged more than 50 colonies per ft² per 24 hr during their stay in the ward, at times when they were the only carriers known to be present. Some rough estimates as to the ventilation rate of the ward suggest that this implies the liberation of 10^6 to 10^7 staphylococcus-carrying particles in 24 hr.

It seems likely, therefore, that the heavy dispersers of staphylococci represent the top end of a continuous distribution. This is compatible with the idea that the degree of dispersal depends largely on the extent of skin contamination with staphylococci, and that the shedding of the staphylococci into the air is due to the continuous desquamation of skin fragments carrying cocci, which either may be transients recently deposited there from the reservoir area in the anterior nares, or may be actually multiplying in or on the skin. The rate of desquamation is presumably related in part to friction of the skin and clothes or other skin areas.

It is, perhaps, remarkable how many bacteria are shed on exercising, even when ⁱ ⁱ subjects

are naked (50). Also, in a few unpublished ob-
servations in coal mines, O.M. Lidwell and I
found that nearly naked miners distributed skin
bacteria into the air in quite large numbers. It may
also seem surprising that carriers liberate as many
staphylococci as they do when it is considered
how relatively scarce staphylococci appear to be
when carriage is determined by swabbing; how-
ever, the area of skin generally examined is very
small, and most methods for the bacteriological
examination of skin are known to be very ineffi-
cient (62).

Factors Influencing Dispersal

Blowers and McCluskey (8) commented that
they have not yet encountered a heavy disperser
of staphylococci among the normal women they
have examined, whereas they found nearly 10%
of men to be dispersers. None of the other studies
has discussed the influence of sex, but, of the 10
heavy dispersers reported by Solberg, 3 were
women, and in general his results do not show any
significant differences between men and women
carriers in the numbers of staphylococci dispersed.
In my ward studies, one of the five heaviest dis-
persers was a woman.

It has been found that treatment of a nasal
carrier of tetracycline-resistant staphylococci with
tetracycline led to an increase in the number of
staphylococci dispersed into the air, presumably
as a result of increased nasal carriage (17), or
possibly as a result of increased skin carriage re-
sulting from a reduction in the normal flora and
a consequent reduction in the fatty acid content
of sebum (18). A similar phenomenon was ob-
served in debilitated or dying patients by Solberg:
an increase in the number of organisms in the
nose and a corresponding increase in the number
shed. M.T. Parker (*personal communication*) has
made a similar observation. One observation that
a concomitant virus infection might increase dis-
persal (16) does not seem to have been confirmed.

A very substantial increase in the number of
staphylococci liberated has been found to follow
the taking of a shower bath (4, 62). The increase
may be 10-fold or more, and the effect persists
for at least 60 min. The mechanism of this increase
is not known, though it is presumed that the
washing in some way allows an increased loss of
the superficial squames.

Within broad limits, clothing makes remark-
ably little difference to the liberation of skin
bacteria, and indeed Speers et al. (50) found that
some of their subjects liberated as many bacteria
when exercising naked as they did when fully
dressed, either in street clothes or in a sterile oper-
ating room suit. The only practicable method so

far described for reducing the rate of liberation
is the use of very closely woven clothing, with a
trouser suit tightly closed at the ankles (4, 8).

Infected Lesions

The discussion to this point has been concerned
with healthy carriers of staphylococci, without
any staphylococcus-infected lesions. As would be
expected, patients with staphylococcal infections
of the skin tend to be especially heavy dispersers
(1, 20, 37). Thom and White (52) found, however,
that there was little dispersal from septic wounds
during the performance of wound dressing, and it
seems likely that the effect of skin lesions is, partly
at least, to increase the load of staphylococci on
the skin. There may also be an increase in the rate
of desquamation, for example, in some patients
with psoriasis, and one such has been implicated
as the source of an epidemic of surgical wound in-
fection in an operating room (32a), though
generally in such patients many of the skin
particles dispersed are too large to remain air-
borne (37). Our own observations (59) indicated
that carriers can be as important as sources of
cross infection as patients with septic lesions.
Burke and Corrigan (13), on the other hand,
found patients with septic lesions to disperse
more staphylococci than healthy carriers; but
they studied only 44 carriers. Patients with chest
infections have been thought from time to time to
be especially dangerous as dispersers (e.g., 44),
but there is little direct evidence on this point.
The possible effect of antibiotic treatment on
dispersal needs to be considered when patients
with septic lesions are being compared with
healthy carriers.

4b. Contamination Resulting from Dispersal

It can thus be concluded that most persons who
carry staphylococci in the nose, all of whom must
from time to time contaminate their skin, liberate
their staphylococci into the air around them. A
small proportion of the carriers are especially
heavy dispersers and give rise to a high level of
aerial contamination. It is not surprising, there-
fore, that there are considerable variations in the
counts of staphylococci in hospital ward air (Fig.
3). The variations in the air counts are, of course,
directly related to the presence or absence of
heavy dispersers in the ward (34). When the air
count in the ward was high, it was virtually al-
ways found that the air staphylococci were al-
most all of one phage type and usually attribut-
able to spread from one person. In the large ward,
there were occasions when two dispersers con-
tributed significantly to the air count, but those
occasions were relatively uncommon (34).
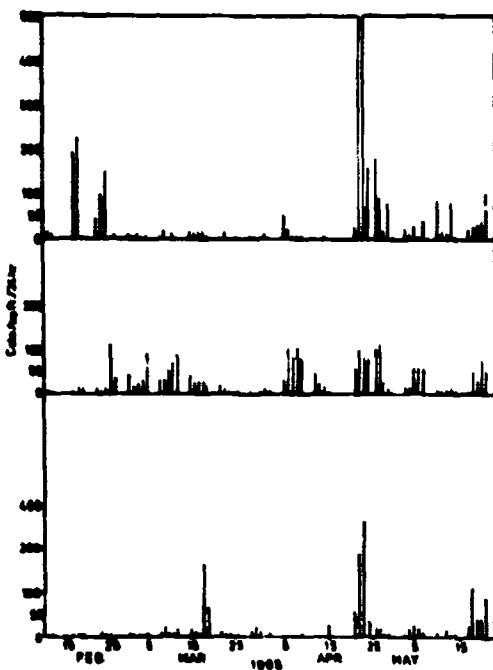
Fic. 3. *Air counts (particles per squa'e foot per 24 hr) of Staphylococcus aureus in three rooms of the divided S. M. H. ward.*

But, as in other situations, the air counts in hospital wards have been found to conform to a log-normal distribution. In Fig. 4, the air counts from the divided ward are plotted as logarithms on a probability scale and are seen to fall close to a straight line. For comparisons between wards, therefore, the median is the most appropriate statistic. Lines depicting the distributions in five different wards are shown in Fig. 5, and the medians from some of them are given in Table 3.

In the S.B.H. open ward, the median count of S. aureus was about 0.1 colonies per ft³. This count was derived from two periods of 2-hr sampling each week, and it might be thought that this could be no more than generally indicative of the total daily exposure of the particles to airborne staphylococci. However, a very similar median and distribution of staphylococcal counts were observed in the open ward at S.M.H., tested by exposure of 12-hr sediment?tion plates.

It is instructive to present the counts in terms of the numbers of staphylococcus-carrying particles that might be inhaled by ward patients in 24 hr. In the two open wards, the median numbers that would be inhaled per day were about 18 and 23 particles; the daily dose exceeded 100 particles on about 15 to 22 °; of days. In the divided wards

at S.M.H. and S.B.H., the median was about 4, and a dose of 100 was exceeded on only about 3 °; of days.

It is interesting to note that in a small series of tests in a ward at the Queen Elizabeth II Hospital, Welwyn, which consists of four-bed bays opening off a wide corridor, the air counts are intermediate between those of the open and the divided wards (data kindly supplied by R. W. Payne). The explanation of these differences clearly demands further investigation, and it is of obvious relevance to the acquisition of nasal carriage of staphylococci, discussed below.

Transfer Through the Air

For a proper understanding of the mode of spread of airborne staphylococcal infection, a knowledge of the s'ze of the airborne particles and of their load of staphylococci is needed. Studies with the size-grading sampler devised by Lidwell (26) indicated that the mean "equivalent diameter" of particles carrying S. aureus was about 14 μ (the "equivalent diameter" is the diameter of a sphere of unit density settling in air at the same rate as the particle in question); the interquartile range was about 8 to 20 μ (38). A much smaller proportion of large particles was observed by Walter et al. (53), using the Andersen sampler, but this is doubtless attributable to the characteristics of that instrument (29). Earlier work by Lidwell and his colleagues (27) indicated that, on the average, airborne staphylococcus
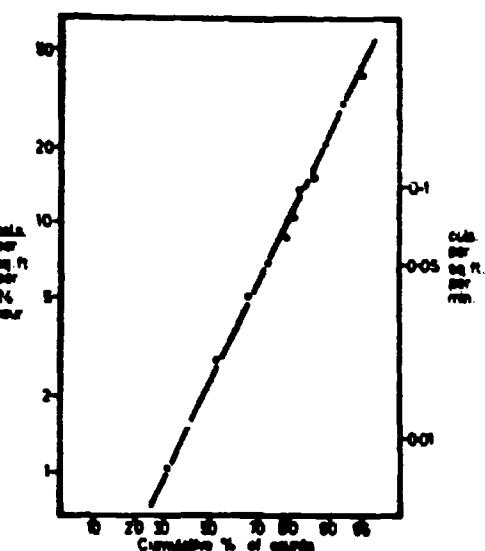

Fic. 4. *Distribution of air counts of Staphylococcus aureus in the divided S. M. H. ward, based on a total of 1,037 12-hr sedimentation plates.*
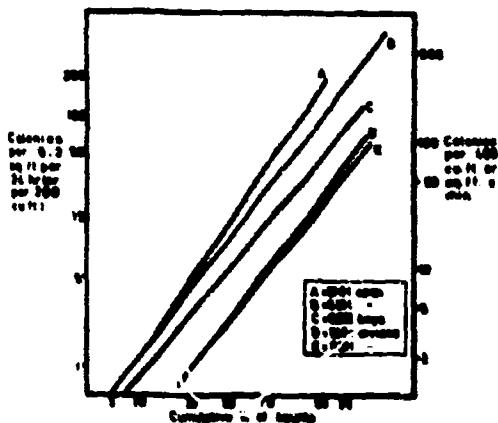
FIG. 5. *Distributions of air counts of Staphylococcus aureus in different wards.* A = S.M.H., 22-bed open ward; B = S. B. H., open wards; C = Queen Elizabeth II Hospital, 4-bed open bays; D = S. M. H., divided ward; E = S. B. H., divided ward.

particles carried about 4 viable cocci, the range being from 6 for the particles greater than 18 μ in diameter to about 1 for those less than 4 μ. These sizes and numbers of bacteria are consonant with the idea that most airborne *S. aureus* cells are associated with desquamated fragments of skin (37).

In normally turbulent air and in a room 10 ft high, particles 14 μ in diameter settle at a rate equivalent to about six air changes per hour, so that 50% of the particles remain suspended for 6 min and 20% for 15 min. Directional air currents of 40 to 50 ft per min are not uncommon in occupied buildings, so that transfer of staphylococci for considerable distances is clearly possible.

In a few studies, in an open surgical ward in which we have found large numbers of staphylococci being dispersed near one sampling point, the mean counts at sampling points about 20 and about 70 ft distant were, respectively, 26 and 11% of the count at the point nearest to the disperser.

When a heavy disperser was present in one of the rooms in the four-room S.M.H. ward, the count in the other rooms has been on average about 5% of that in the room with the disperser. Lidwell and his colleagues (27a) have studied a ward divided into nine rooms and found that the count in rooms other than that containing a source of staphylococci is about 10% of that in the source room.

In studies of staphylococcal infection in a surgical operating room, Shooter et al. (46) demonstrated what appeared to be aerial transfer over

the distance of 90 ft that separated the wards from the operating room.

The actual extent to which staphylococci can be conveyed within a ward or between rooms must depend on the local circumstances of structure, site, and ventilation, but enough has been said to show that aerial conveyance over considerable distances is quite possible.

The aerial route is not, of course, the only way by which staphylococci can be conveyed from one room to another, and hospital routines commonly prescribe quite elaborate rituals for dealing with potentially infected dust on floors, shoes, trolley (cart) wheels, and the like, which is thought to generate secondary airborne spread. But, though many workers have estimated the bacterial content of floors, few have made any useful studies of actual transfer by this route (see 53).

Viability in Air

There is a considerable amount of laboratory work to show that staphylococci commonly survive in the dried state for periods measured in days or weeks. Whether there is any significant alteration in their infectivity on storage is not certain. Indications of some loss of infectivity were obtained by Hinton et al. (23) and by Taylor et al. (51); other workers have found no such effect (e.g., 28, 42). Noble's (33, 36) experimental work in animals has suggested that any effect on infectivity from desiccation is limited to an extension of the lag period and that, if staphylococci are protected from body defenses immediately after introduction into the tissues, they are as virulent as fresh organisms.

ACQUISITION OF AIRBORNE STAPHYLOCOCCI

There are two important ways in which airborne staphylococci might infect patients in hospitals: by inhalation or by settling directly into some susceptible area, such as a wound, or onto instruments or dressings that subsequently come into contact with the wound. Inhalation infection may occur anywhere and at any time; sedimentation infection is of particular importance in operating rooms and treatment rooms where surgical wounds are exposed, often for long periods of time. It will be convenient to deal with sedimentation infection in operating rooms first.

Operating Room Infection

Airborne transfer from without. Recent studies of air hygiene in surgical operating rooms date largely from the work of Bourdillon and Colebrook (10) in a Burns Unit treatment room, but it was the application of their work to the control of a high incidence of postoperative staphylococ-

cal wound infection by Blowers et al. (6) and by Shooter et al. (46) that brought the subject to general attention. Shooter et al. estimated that, in an 8-month period, the incidence of operating-room infections was 9% of 427 wounds; 0.07 particles per ft³ containing *S. aureus* were found in samples from the air during operations. A simple alteration of the ventilation so as to generate a positive pressure in the operating room and exclude staphylococcus-contaminated air from the wards was followed by a substantial reduction in the general air bacterial count (the number of *S. aureus* was not reported), and by a reduction to less than 1% in the incidence of sepsis of presumed operating theater origin in 532 wounds. It is reasonable to assume that this reduction in sepsis was attributable to a reduction in the number of staphylococci settling from the air into the wounds and onto the sterile instruments and equipment. No other investigation has been reported in which alteration of the ventilation was the only change made, but the published report of Blowers et al. (6) and subsequent unpublished experience (Blowers, *personal communication*) supported the general idea that the prevention of contamination of operating room air with bacteria from other parts of the hospital by the introduction of positive-pressure ventilation has often been associated with a reduction in the incidence of postoperative sepsis. Blowers and Crew (7) recorded a mean *S. aureus* count of 0.6 colonies per ft³ in an exhaust-ventilated operating room, compared with 0.03 colonies per ft³ in a plenum-ventilated operating room.

Locally generated aerial contamination. The work just cited concerned contamination of operating room air with staphylococci from other parts of the hospital, drawn into the operating room by air currents. It is this form of transfer that is controllable by positive-pressure ventilation. But aerial contamination can also be generated within the operating room, either by disturbance of the patients' bedclothes and drapes or from the skin of the operating room personnel, as discussed already.

Air counts and infection rates. The bacterial count observed in the air of an operating room is clearly the sum of that produced by infiltration of contaminated air from without and that generated locally. It would be of great value for the monitoring of operating room hygiene if it were possible to relate the staphylococcal (or even the total bacterial) count in the air to the risk of postoperative sepsis. The difficulties of deriving such a relationship are, however, very great. The incidence of wound sepsis is in any case generally very low perhaps between 1 and 5%. Only a

part of the septic cases are infected during operation, and this portion is difficult to estimate, and in any case is not all attributable to sedimentation of airborne staphylococci. In addition, the numbers of staphylococci actually settling onto susceptible areas are so small as to be difficult to measure.

Burke's (11) study is in many ways the most detailed available. By using a very sensitive technique he was able to recover *S. aureus* from 46 of 50 wounds examined at the end of operation; most wounds yielded two or more different strains, and the mean number of viable units of staphylococci was 14 per wound. Potential sources for the staphylococci found in the wounds were: air, 68%; carrier site on patient, 50%; hands or nasopharynx of the surgical team, 20%. (In some cases, there were two or more potential sources.) Only 2 of the 50 wounds developed any clinical sign of postoperative infection; the rate for wounds that had not been carefully washed out for bacteriological examination was not presented.

In a comparable study of the sources of infection in 35 patients who developed wound sepsis apparently resulting from operating room infection, Bassett et al. (3) thought that a member of the surgical team was concerned in 31%; and the patient himself in 17%, the source of the remainder being untraced.

There are several other published studies in which an attempt was made to relate postoperative infection to airborne staphylococci found in the operating room (e.g., 24,53,60), but they do not allow easy summary. The general impression is that staphylococci of the type responsible for postoperative infection were rarely found in the air, but this may well reflect the very small air samples generally examined.

In general, it appears that, in reasonably well-ventilated operating rooms with good staff discipline, the *S. aureus* count is of the order of 0.01 to 0.05 colonies per ft³, in a series of operating rooms, we have observed a mean settling count of about 0.01 colonies per ft³ per min, while an American cooperative study reported a count as low as 0.001 colonies per ft³ per min. (33).

The operating room ought to be a situation in which it would be possible to determine the average infecting dose of staphylococci for man. Taking a figure of 0.01 colonies per ft³ per min, and assuming an effective target area of 1 ft³ (to include instruments, etc.) and a duration of operation of 2 hr, a frequency of operating room infections of 1%, would imply that the 1% infective dose is about 1 staphylococcus-carrying particle. But, to put any real meaning into the

figures, we need to measure the air count and the sepsis rate in a far greater number of patients than has yet been attempted and to carry out at the same time elaborate bacteriological cultures on the patient himself and on the ward to try to assess the relative importance of routes of transfer other than air. And we should remember Burke's (12) thesis that sepsis is often determined largely by the condition of the actual tissue on which the staphylococcus alights, and on the state of the patient; if his observations are generally applicable, there are usually plenty of staphylococci.

Infection in Wards

There is ample documentation of the rate at which both newborn infants and adult patients become nasal carriers of the prevalent staphylococcus in many hospital wards (56). It was reasonable to postulate in the first place that these staphylococci reached the nose by way of the air. Evidence has been sought on this point in several ways—by examining the order in which different parts of the body are colonized, by attempting to interfere with transfer by one route or another, by trying to identify the source of the staphylococcus more precisely, and by relating the acquisition rate to the staphylococci found in the air.

The most precise investigations in this field concern newborn infants. It was shown, first, that the umbilicus and abdominal skin are generally colonized before the nose (25, 48). Second, Rammelkamp and his collaborators showed that a nurse carrier only conveyed her staphylococci to infants if she handled them (61), and later that the colonization of the infants could be delayed by increasing the precautions against contact infection (31, 32). With very strict precau-

TABLE 1. Primary site of colonization in adults[a]

Carrier sites, etc., positive before nose for staphylococci of same phage type	No. of patients who became nasal carriers in ward		
	Nose positive twice or more	Nose positive once only	Total
None	19 } 25	25 } 28	44 } 53
Clothing only	6	3	9
Dressing or wound	7	5	12
Hand or other skin site	14	2	16
Total	46	35	81

[a] Patients swabbed daily; apparent acquisitions on first 3 days of hospital stay, and acquisitions of untypable strains, excluded.

tions against cross infection, the rate of acquisition of staphylococci was reduced from 43 to 14%; the latter infections were assumed to be due to aerial transfer. The relative unimportance of inhalation infection in newborn infants is perhaps hardly surprising, when one considers that the infant has a minute volume of only about 500 ml (about 0.02 ft³), and that he has to be handled frequently, usually by nurses who handle a good many other infants. But a 14% acquisition rate in a 4-day hospital stay is equivalent to some 3 to 4% per day, which is of the same order as observed in adult wards.

With the evidence from the newborn infants in mind, it is pertinent to ask whether the nose or some skin site is the first area to be colonized in the adults who acquire staphylococci in hospitals. It is obviously more difficult to obtain evidence on this for the adult than for the infant, but in a study of surgical patients (22) R. A. Henderson examined swabs daily from the nose, skin of the hands, skin near the wound site, wound, bedclothes, and environment (Table 1). Some 20% of the 81 patients who became nasal carriers yielded staphylococci of the relevant phage type from one or other of the two skin sites before its appearance in the nose, and a further 15% had yielded the staphylococci from the wound. In the remaining 66% of acquisitions, the nose was the first site on the patient found to yield the staphylococcus. Two important provisos have to be entered here: there was a striking dominance of staphylococci of one phage type among the acquisitions in the ward, which means that there is a serious risk of regarding as related two independent acquisitions; and the area of skin examined was very small and perhaps not representative. Additionally, even skin or clothing contamination might result from airborne transfer, which need not operate only to give inhalation infection. However, the evidence, such as it is, does not contravert the idea that direct inhalation infection is important in the acquisition of the nasal carrier state in adults.

In our recent study at St. Mary's Hospital, 53 patients were observed to acquire nasal carriage of S. aureus while in the ward. The same phage type had been recovered from the air prior to its recovery from the patient in 64% of cases (Table 2). In this ward, there was no marked dominance of one type, and indeed 25 types are represented among the 53 acquisitions. Again, this is not formal evidence that the nasal carrier state was acquired by inhalation of cocci, but it is consistent with such an explanation.

Further evidence for the importance of aerial transfer comes from studies of different ward

structures. In an open 22-bed ward we found that separation of patients by the full length of the ward (about 50 feet) only reduced the rate of acquisition of staphylococci by about one-half, as compared with the acquisition rate for a patient in a neighboring bed (59). At the other extreme, very low nasal acquisition rates have been found in patients nursed in single rooms opening to fresh air, that is, when the chance of aerial transfer from one room to another is very low indeed (40). There also appeared to be very little spread of tetracycline-resistant strains from patients nursed in isolation rooms fitted with exhaust ventilation, and the acqusition rate for such strains was greatly reduced in a ward in which all patients harboring such strains were isolated (59).

In adult patients, there are technical difficulties in recognizing the acquisition of nasal carriage that are not present with infants, since truly per-

TABLE 2. Number of patients showing apparent acquisition of nasal carriage in relation to previous air exposure (S. M. H.)[a]

| No. of weeks nose negative for the acquired staphylococcus before acquisition | Staphylococci of acquired type— | | Total |
	Found in air previously	Not found in air previously	
1	15 (9)[b]	11 (8)	26 (17)
2	9 (6)	2 (2)	11 (8)
3	3 (2)	2 (2)	5 (4)
4	3 (1)	2 (2)	5 (3)
5+	4 (3)	2 (1)	6 (4)
Total	34 (21)	19 (15)	53 (36)

[a] An additional 13 (9) patients were found on admission to the ward to be carriers of a staphylococcus previously found in the air and so may well have acquired their nasal carriage in the ward.

[b] Numbers in parentheses give patients carrying the acquired strain on one occasion only.

sistent carriers may fail to yield staphylococci on some occasions. However, since carriage of tetracycline-resistant staphylococci is even now relatively rare (at least in Britain) in people outside hospitals, such strains form a convenient indicator of hospital acquisition. Some rates of acquisition of tetracycline-resistant staphylococci in various wards are presented in Table 3. Unfortunately, data for tetracycline resistance of staphylococci isolated from air samples in these wards are not available, so it is only possible to compare the ranking of the wards with respect to the two parameters. The very limited results suggest that the acquisition rate was higher in the wards with the higher counts of air staphylococci. For various technical reasons, it has not yet been possible to test directly the relation of the acquisition rate to the exposure to particular staphylococci, though this clearly needs to be done.

Minimal infective dose. If we are to understand the epidemiology of airborne infection, we must know the minimal dose of microbes ordinarily needed to effect colonization or infection; this number is not known, but it is so important that it seems justified to indulge in some extrapolation from the few figures available. Shinefield and his colleagues, in their investigations of bacterial interference, found that they could set up a carrier state in the nose of 50% of newborn infants by the inoculation of between 200 and 400 cocci. As noted already, most airborne staphylococcus-carrying particles appear to contain no more than one to six viable cocci.

In experimental infections, it is generally found that the relation between dose and attack rate is not linear, but conforms to an S-shaped curve. For extrapolation to be possible, it is therefore necessary to apply some transformation to the data, e.g., to plot the logarithm of the dose inoculated against the probit of the percentage attack rate. This has been done in Fig. 6 for the data obtained by Shinefield and his colleagues (43, supplemented by a personal communication from

TABLE 3. Acquisition of nasal carriage of tetracycline-resistant Staphylococcus aureus in relation to daily exposure to airborne staphylococci

Reference	Type of ward	Median exposure (particles/24 hr)	Acquisition rate (per cent per day)
Williams et al. (59), Noble (34)	22-24 bed open, S. B. H.	18	0.7
Shooter et al. (47)	22 24 bed divided in two parts, S. B. H.	9[a]	0.6
		8[a]	0.3
Williams (in preparation)	14 beds in 4 rooms, S. M. H.	4	0.3
Lidwell et al. (27a)	30 beds in 9 rooms, S. B. H.	4	0.1

These values are estimates based on mean counts provided by O. M. Lidwell, converted to medians on the assumption that the distribution was similar to that in the earlier S. B. H. studies.
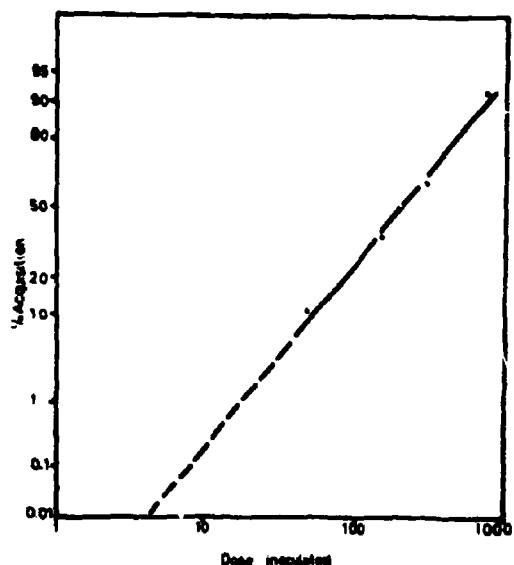
Fig. 6. *Relation between dose of staphylococci inoculated into infants' noses and acquisition rate for nasal carriage. Based on figures kindly supplied by Henry R. Shinefield.*

Dr. Shinefield), and the points lie very close to a straight line. Extrapolation of the line back would suggest an attack rate of about 0.02% for a dose of five cocci. The observations of Shinefield et al. were made on newborn infants who are presumably more susceptible to staphylococcal colonization than adult subjects, but, in the absence of any other figures, the calculation may be worth pursuing.

The data in Fig. 5 suggest that the median number of staphylococcus-containing particles inhaled in the S.B.H. wards may have been about 18. Each of these particles probably contained, on the average, about 4 viable cocci, so that the total daily dose inhaled could be estimated at about 70 cocci; if the dose-response relation observed by Shinefield were applicable to the adults, this dose might be expected to generate a "take-rate" of just over 10% per day if all the inhaled particles co-operated to set up the carrier state, or 0.16% if they acted independently. Unfortunately, we do not know how many of the airborne staphylococci were tetracycline-resistant, but the apparent acquisition rate for tetracycline-resistant strains was about 0.7% per day.

In the S.M.H. divided ward, the median dose of sensitive and resistant staphylococci inhaled was about 16, which on Shinefield's figures would indicate a take-rate of 0.6%, or less than 0.01% if all the particles acted independently; the actual rate

of acquisition of tetracycline-resistant strains was 0.3% per day.

These and some other similar data are presented in Table 3. Although quite insufficient to indicate a clear relation, they suggest that the staphylococcal acquisition rate in different wards may well be related to the air count. In fact, the acquisition rates in the wards are, considering the amount of extrapolation involved, clearly of the same order as those predicted from Shinefield's figures. But at least these calculations clearly indicate that there is no wild improbability in the idea that the acquisition of the nasal carrier state in surgical patients results from the inhalation of such airborne staphylococci as can be shown to occur in the wards. The number of complicating factors in any precise analysis is formidable.

In the first place, as already noted, the figure for a median bacterial count conceals enormous variations, and we clearly need to know whether a short exposure to a large number of airborne staphylococci is equivalent to a more prolonged exposure to smaller numbers. A second complication arises from the fact that staphylococci appear to vary in their ability to colonize the nose (57), so that there is reason to think that inhalation of large numbers of cocci of some strains may be less effective in setting up the carrier state than inhalation of others. The third complication arises from differences in the recipients. The phenomenon of bacterial interference, studied in detail by Shinefield et al. (43, 43a) in infants, almost certainly operates in adults also. Several workers have shown that patients admitted to hospital as carriers of *S. aureus* are less liable to acquire hospital strains than patients admitted as noncarriers (e.g.,58). The fact that, at least in open wards, patients treated with antibiotics acquire hospital staphylococci in the nose more often than those who are not (e.g., 39) is presumably another example of the same phenomenon, which was well demonstrated experimentally by Boris et al. (9). At the same time, antibiotic treatment probably prevents nasal acquisition in other patients.

Relevance of Nasal Acquisition

In the operating room, we must assume that, whatever the dose-response relation, the aerial transfer of staphylococci to the wound itself is potentially important. It may be asked whether there is any corresponding relevance in the nasal acquisition of staphylococci in the wards. There seem to us to be two ways in which the nasal spread is important.

In the first place, it appears that, at least in some circumstances, nasal carriage of staphylococci predisposes to postoperative infection

(58). There has been some discussion on the significance of these observations (3, 22, 30), but scrutiny of the records of a considerable number of patients leaves no doubt in my mind that the phenomenon is real, even if not generally so frequent as suggested by our original observations.

But nasal carriage is also relevant in that it seems to be the mechanism by which the endemic staphylococci persist in the hospital. Such persistence can often be for a long period. For example, at Saint Bartholomew's Hospital we observed the spread of a staphylococcus of phage type 75 77 which continued from the start of the study in one ward in February 1959 until the end of January 1960. During this period of 1 year, there were only 39 days when there was not present a patient who was either known or reasonably presumed to be a carrier of the strain. A total of 23 patients were infected with the strain, but only 6 of them had any clinically infected lesion.

CONCLUSION

The commensal association of staphylococci with man is universal (56) and to a large degree harmless. The transfer from one individual to another must, under ordinary circumstances, very often be by direct or indirect contact. But ability to disperse *S. aureus* into the air in large numbers is a characteristic sometimes temporary and sometimes persistent of a number of healthy people, and wherever we go indoors there is a chance that we shall inhale staphylococci. [A few observations in two Post Offices in London have given an average sedimentation count of 0.01 colonies per ft² per min, a figure quite similar to that for hospital wards (J. Corse, *personal communication*)]. But it is only in hospitals that any detailed study of the processes of transfer has been made.

Airborne transfer in hospitals gains its special significance from the fact that, if this route is actually operative, a single disperser is potentially able to infect a considerable number of other patients, who need not be confined within the same room, or even perhaps on the same floor; and the transfer of infection cannot be contained by ordinary methods of asepsis.

The evidence that has been reviewed seems to leave little doubt that airborne transfer can be of importance. It suggests that the acquisition of nasal carriage of *S. aureus* by patients nursed in hospital wards can be explained if the dose-effect relationship determined experimentally in infants is approximately applicable to adults. If the results obtained in the studies reviewed can be confirmed elsewhere, we should have a rational

basis for assessing one aspect of hospital hygiene in relation to the prevention of staphylococcal infection. We still lack, however, a precise measure of the relative part played by this airborne spread in the etiology of staphylococcal hospital-acquired infection generally.

To take surgical wound infection as an example, we have to recognize that infection can be derived from: (i) staphylococci carried by the patient on admission to hospital; (ii) staphylococci that the patient has come to carry in the nose and on the skin after admission, which subsequently enter the wound; and (iii) staphylococci that reach the wound directly without the prior intervention of the nose or skin carrier state. It appears that aerial transfer plays a major part in the second of these categories and a part sometimes major and sometimes minor—in the third. But we have insufficient precise evidence on the relative importance of the three categories themselves. The proportion will clearly differ greatly from one hospital to another, and within one hospital, from one sort of surgical operation to another, and from time to time.

The challenge with which we are faced is to provide much more firmly based estimates of the relative frequencies in these categories and the factors that determine them. The practical justification for attempting such an analysis is that it can provide the only basis for judging how best to construct and ventilate hospitals. And the fundamental difficulty of performing the analysis is that in any hospital, where the analysis would be practicable, the overall incidence of infection is probably no more than 1 to 2%, and this small proportion must be distributed over all the various routes and sources.

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Discussion

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Dr. Williams has presented a perceptive review of our knowledge of the occurrence of *Staphylococcus aureus* in the air of hospital wards and surgical operating rooms and of its spread to patients. It is impressive how much work has been reported during the past decade and what a

major contribution Dr. Williams and his colleagues have made to the total body of this knowledge. He has been penetrating in his analyses and careful to limit his rather guarded interpretations and conclusions to areas in which he has substantial microbiological data to support his long experience.

The present discussant has neither these assets nor limitations. As a general practitioner of epidemiology and an early convert to a recognition of the importance of airborne infection in some diseases, I believe I can best contribute by discussing certain broader issues that may be related not only to the subject of staphylococcal infection, but also to the whole purpose of this Conference.

In his introductory paragraph, Dr. Williams points out, "It is a characteristic of the airborne route of infection—in contrast to transfer by food or water—that whenever there is the possibility of aerial transfer there is almost always also the possibility of transfer by other routes." Obviously Dr. Williams was thinking about staphylococcal infection in hospitals, where his concept is crucially important, but, considering the generalization as stated, it needs considerable qualifications.

Until the mid-1940's, our concepts of airborne infection were vague. In fact, to most epidemiologists and microbiologists, the term still carried the stigmata of miasms and malarias of the pre-bacteriological era. During the past 20 years, however, our concepts of airborne infection have become increasingly sharp and distinctive, owing in no small part to the studies conducted by the U.S. Army Biological Laboratories and the Microbiological Research Establishment in the United Kingdom.

The clinical, pathological, and epidemiological patterns of primary histoplasmosis, coccidioidomycosis, inhalation anthrax, primary pulmonary tuberculosis, Q fever, tularemic pneumonia, pneumonic plague, and a host of laboratory-acquired infections leave no basis for confusion as to their airborne origin. In these diseases, the portal of entry is the terminal bronchiole or alveolus of the lung. Infection arises by the inhalation of small particles 1 to 3 μ in size. The primary pathology appears in the periphery of the lungs or the mediastinum. Epidemiologically, there is a history of direct exposure to an aerosol or to gross aerial contamination of large rooms, whole buildings, and at times the outdoor atmosphere. The airborne character of these diseases can no longer be rationally disputed. When these infections occur by other routes, for example, scrofula from tuberculous milk, tularemia from a tick bite, or bubonic plague from a flea, the clinical and epidemiological patterns are distinctive.

Turning our attention to infections in which the portal of infection is the respiratory epithelium of the nose or the tonsillar tissue of the nasopharynx, the problem of differentiating possible multiple routes of transfer does ise. It has long been a challenge to the epidemiologist to distinguish among four related, but distinct, modes of spread: (i) direct contact, as in kissing, (ii) indirect contact, as in the use of contaminated surgical instruments; (iii) droplet infection with direct impingement on the face, mouth, or conjunctiva; and (iv) airborne infection from the inhalation of suspended droplet nuclei or infectious dust particles that have travelled some distance through the air.

In is interesting and pertinent to isolate staphylococci from the air of wards and operating rooms and to show that these organisms have retained their virulence. Likewise, it is highly suggestive to isolate staphylococci from the anterior nares of postoperative patients. But such findings by themselves are insignificant. The human nose is an excellent filter, especially for larger aerosols. The isolation of a few staphylococci from the nose, particularly if only on a single occasion, may be of little or no significance. The infectivity of a dried bacterial particle in metabolically suspended animation almost certainly is less than that of a droplet. Such a moist particle may carry actively metabolizing bacterial cells along with toxic products, enzymes, and receptor substances that may greatly facilitate invasion of tissue and enhance the competitive position of the microorganism vis-a-vis the body's still-to-be-mobilized defense mechanisms.

What is essential and necessary evidence is the demonstration that the elimination of one or more of the means of spread, keeping the remaining ones constant, radically and consistently reduces the incidence of actual disease. The importance of contact, both direct and indirect, was established so long ago that it now tends to be forgotten or ignored. The development of aseptic surgery proved that contact was the dominant mode of spread of surgical sepsis and that airborne infection was of minor consequence. Application of the same aseptic principles to hospital management permitted the development of the modern pediatric hospital and contagious disease services, again establishing the dominant importance of contact-infection.

It is more difficult to distinguish precisely the role of droplet infection, because it is so closely associated with direct contact. The tendency of many persons to equate droplet infection with the airborne route rather than with contact is, in my judgment, ill-advised. The control of droplet infection calls for strict personal hygiene, wearing of masks, and other individual measures regularly

associated with aseptic techniques rather than for the engineering methods applicable to the control of aerosols and dust.

Some rather heroic efforts have been made to control airborne infection in pediatric and surgical wards and operating rooms with controlled ventilation and ultraviolet irradiation. It is a safe generalization to say that, the more carefully controlled these experiments, the less impressive the evidence in favor of airborne infection has been. As emphasized by Dr. Williams, when strict asepsis is enforced to minimize contact and droplet infection, the rate of hospital-acquired infection is reduced to well below 5%, and may reach 1%. It is exceedingly difficult to prove whether this low residual rate results from airborne infection or from failure of the aseptic techniques to eliminate all contact.

Dr. Williams has drawn guarded conclusions regarding the role of airborne staphylococcal infection in hospitals. He has emphasized the multiple sources of infection and modes of spread. He admits that "we have insufficient precise evidence" on their relative importance. The epidemiological evidence, also far from precise and based on more general considerations, supports his caution.

Discussion

Viability of Hospital Staphylococci in Air

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The role of airborne transmission in the spread of staphylococci in hospitals (3) has not been definitively established. Though several authors studied the survival of staphylococci in air and on surfaces (1, 2, 4), exact knowledge of the survival of "epidemic" and "nonepidemic" strains is scarce.

The survival of a number of strains (for description, see Table 2) in air of differing relative humidity (RH) was studied by spraying with a direct spray (FK8) in a static system (4,000 liters) and sampling 10 liters in a slit sampler on blood-agar plates (Fig. 1a). The reference strain no. 1600 (isolated from a nose swab, phage type 187) showed a low decay rate at 50% RH and an increased decay rate at high RH (Fig. 2). This effect was generally found with most strains. During the experiments, it became clear, however, that by varying the growth medium, the age of the culture, the suspension medium, the method of aerosolization, and the composition of the collection plates, any desired result could be obtained. Decay curves could be logarithmical or curved, and the effect of relative humidity could be very marked or nonexistent. This variability was specially marked at high relative humidities, and less so below 50%. The late

TABLE 1. *Survival in air of strain isolated in hospitals compared with reference strain 1600 (90°; RH)*[a]

Strain	Phage type	$K \times 10^4$	K/K_{ref}
1600	187	87, 81, 79, 89, 85, 95, 82, 96, 85 95, 76, 97	1.00
1	80/81	95	1.09
7	80/81	74	0.94
17	80/81	77	0.81
18	52/80/81	73	0.77
3	52/80/81	77	0.89
8	52/80/81	70	0.80
1330 A	52/80/81	76	0.94
1451 A	52/80/81	70	0.79
2	NS I-III	84	0.97
1330 B	NS I III	87	1.07
1451 B	NS I-III	76	0.89
4	III	79	0.98
10	III	102	1.15
11	III	92	1.03
16	III	108	1.14
15	83 A	114	1.27
13	III type A	117	1.38
9	II	79	1.00

[a] Three strains were tested daily together with the reference strain. K/K_{ref} was calculated with the K_{ref} observed on that day. Calculation based on the mean of K_{ref} reduced the differences.

appearance of small colonies on collection plates at high RH (Fig. 1b) indicated that many organisms were damaged and started growth only after a long lag.

An instance of variability with culture age is given in Fig. 3 and 4. Figure 3 gives the growth curve of strain 1600 in nutrient broth (Difco). At the indicated times (↑), the number of single organisms and of clumps of two, three, or more organisms was determined, or a sample of the
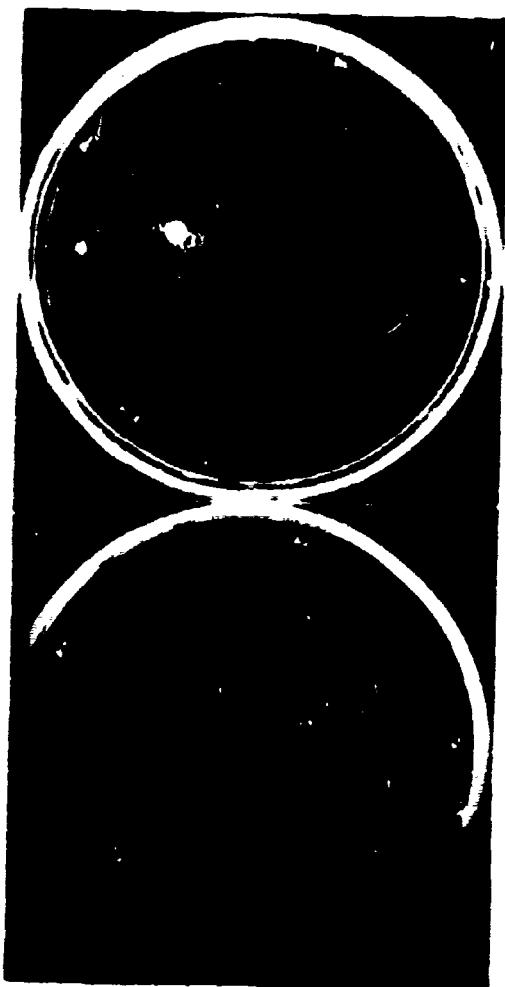

Fig. 1. Colonies of staphylococci (strain 1600) recovered from aerosols in air of 20.0 C on blood-agar plates in a slit sampler. The four sectors represent 20-min (10-liter) samples 40, 45, 50, and 60 min after aerosolization; (a) 40% RH, uniform colonies; (b) 80% RH, increasing numbers of small colonies due to metabolic damage in later samples.
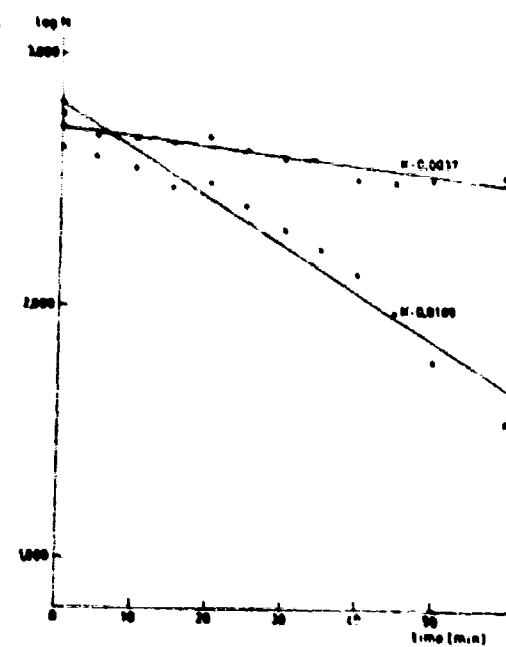
Fig. 2. Decay curves of Staphylococcus no. 1600 at 39% RH (●) and at 75% RH (○).
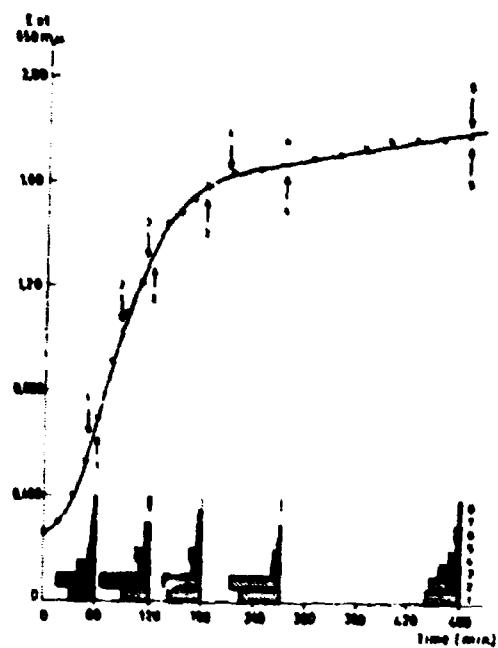
Fig. 3. Growth curve of strain 1600, with frequency distribution of single organisms and clumps of various sizes shown at the bottom of the figure (right-hand scale). At the indicated times (↑), samples were diluted and aerosolized (compare with Fig. 4).

culture was diluted ($\frac{1}{2}$) and aerosolized. Figure 4 gives the corresponding decay curves at 70% RH.

Finally, we arrived at an experimental procedure that gave reproducible results and decay curves which were straight on a logarithmic scale (Fig. 5) at all RH levels. The strains were inoculated from stock cultures into 10 ml of meat infusion broth and incubated for 8 hr at 37 C. This culture was diluted 1:500, and a standard droplet (0.03 ml) was inoculated into 20 ml of meat broth which was incubated for 16 hr on a turntable at 37 C. This culture was diluted with meat broth to an extinction of $E = 0.180$ (Unicam). A further dilution of 1:100 was made with meat broth. This suspension containing about 6×10^5 viable particles per cubic meter was sprayed with a direct spray (FK 8) with 5 atm of nitrogen (0.5 ml in 4,000 liters). Collection was on double-layered blood-agar plates with 10% sheep blood in a meat broth base. Important features seem to be the incubation under slight

agitation, the use of metabolically inert organisms after 16 hr of growth, omission of centrifugation and washing, and the use of a direct spray. Recoveries were between 40 and 90%. At low

FIG. 5. Decay curves of staphylococci at 90% RH Standardized experimental procedure. Curves calculated with least squares. Strains 1, 2, and 3 from Table 1. No. 4 = strain 1600.

TABLE 2. Survival in air of strains which had caused epidemic events in hospitals, compared with reference strain 1600 (90% RH)

Strain	Phage type	$K \times 10^3$	K/K_{ref}
1600	187	85	1.00
3827	80/81	87	0.90
3828	80/81	78	1.03
3829	80/81	77	1.01
3830	80/81	76	1.00
3822	52/52A/80/81	74	0.75
3826	6/7/47/53/54/75/83A/81	76	0.79
3824	6/53/83A	91	0.96
3825	NT type A	84	0.88
3823	83A	91	0.96

FIG. 4. Variance of decay curves with culture age (70% RH).

RH, they were slightly higher. Differences between strains were negligible. In Table 2, the mean recovery was 48%, with $\sigma = 24\%$.

The results with a number of strains are given in tables 1 and 2 in terms of total decay rate K $(K = \frac{\Delta \log N}{\Delta t}$, where N = number of organisms and t = time in minutes). Physical fall out in the system, as tested with spores and with fluorescein, was below $K = 0.002$, but was not subtracted. No obvious difference was observed between strains of various phage types, all isolated from patients (noses, lesions, etc.) or between the strains received by the courtesy of M.T. Parker from the Central Public Health Laboratories at Colindale, London, England, which had given rise to epidemics (Table 2).

The data seem to indicate that "epidemic" strains do not survive better than other strains

in air. Considering the difficulties in standardization of experimental conditions and our lack of knowledge of the factors causing the reproducibility, it remains possible that differences between strains are masked by the procedure used.

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Experimental Epidemiology of Coccidioidomycosis

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INTRODUCTION

Coccidioidomycosis, a highly infectious but noncontagious disease caused by the dimorphic fungus *Coccidioides immitis*, is limited mainly to the desert regions of southwestern North America and the Grand Chaco-Pampa region of South America (Fig. 1).

This disease, primarily a respiratory infection, manifests itself over a wide range of severity, from acute bronchitis or pneumonia in about 40% of the infections to subclinical or nonsymptomatic disease in approximately 60% of the instances (10, 30). Occasionally, in a few infections (0.5 to 2%), extrapulmonary dissemination takes place, resulting in a fatality rate of approximately 50% in the disseminated cases (Fig. 2). The disease is of great economic importance in the endemic areas, from the standpoint of both human and

canine infections (16, 25). As an example, Hugenholtz (19a) has reported that at Williams Air Force Base, near Phoenix, Ariz., the cost of man-hours lost and of hospitalization due to coccidioidomycosis approaches $70,000 per year. Other Air Force bases in the area have estimated the cost of these infections at $50,000 to $100,000 yearly. When the number of Air Force Bases, Army installations, etc., in the Southwestern United States is taken into consideration, this could amount to a significant cost to the government.

Respiratory exposure results from inhalation (20) of arthrospores of the saprophytic phase, which grow in the soil and are disseminated by wind during dust storms (12, 30). Although *C. immitis* can readily be isolated from the soil (5-9, 15, 23, 33), isolation by means of air samplers is

FIG. 1. *Endemic areas of coccidioidomycosis in North, Central, and South America* (10). (*Courtesy of M. J. Fiese.*)

extremely difficult. Consequently, not much is known concerning the size of the infectious dose in nature.

The purpose of the present study was an attempt to determine the infectious dose by the use of laboratory animals as "biological air-samplers." As a baseline for the study, the U.S. Army Biological Laboratories has a large amount of data (2-4, 28) on the pathogenesis of coccidioido-

mycosis in monkeys and in dogs (serology, histopathology, X ray, etc.) exposed to graded respiratory doses of 10 to 80,000 *C. immitis* arthrospores.

In our experience, the dog was as susceptible to the disease as the monkey, but was more resistant to its effects, because of its ability to maintain a blood supply to the lesions for a longer period of time and because of a faster and more prolific collagen response to the presence of the organism

PATHOGENESIS OF COCCIDIOIDOMYCOSIS
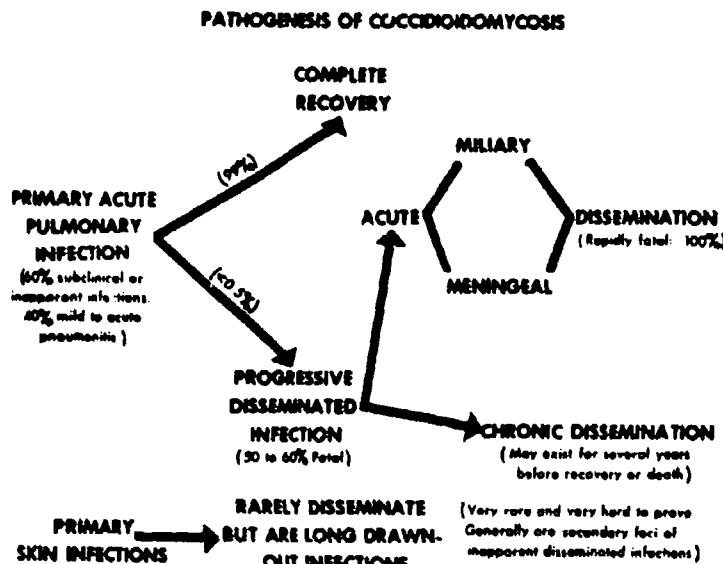
FIG. 2. Schematic summary of types of infection caused by Coccidioides immitis.

(3). It was postulated that the monkey is most susceptible to the ravages of the disease, that the dog is least affected, and man is somewhere on the scale between the species, but probably much closer to the dog than to the monkey. Moreover, other experimental studies in dogs by Reed (26) and Hugenholtz et al. (13) have indicated that the pathogenesis of coccidioidomycosis in man and dogs is very similar; the only real difference lies in the bone lesions, which are more destructive in man and more proliferative in dogs (16, 25).

With this type of data available, it seemed logical that observations on monkeys and dogs exposed in the open, in an endemic area, might be compared with observations of animals receiving known experimental respiratory doses, thereby resulting in a valid estimation of the infectious dose of C. immitis received by man in nature.

CHOICE OF LOCATION FOR NATURAL EXPOSURE OF ANIMALS

The endemic areas of coccidioidomycosis and the climatic and geophysical conditions necessary for growth of C. immitis in the soil have been firmly established. Among the major contributions in this area of study have been: (i) the very thorough epidemiological studies of C. E. Smith and his co-workers (29-32) and Palmer et al. (21), using coccidioidin skin hypersensitivity in man to define the endemic areas, and to correlate rainfall and dry, dusty atmospheric conditions with seasonal morbidity rates (30); (ii) similar studies by

Maddy et al. (17, 18) through widespread skin testing of home-grown cattle; (iii) Hugenholtz's study of the optimal climatic factors for growth of C. immitis in the soil (12); (iv) the extensive soil studies by Egeberg (6, 7) and others (8, 15, 22), associating soil types and salinity of the soil at various seasons with optimal growth conditions for C. immitis; and (v) a demonstration of the close association of the boundaries of the Lower Sonoran Life Zone (Fig. 3) with those of the known endemic areas of coccidioidomycosis, by Maddy (17).

These investigators have suggested that ideal conditions for the fungus to maintain itself in the soil include an arid or semiarid climate, hot summer months, mild winter temperatures, light, slightly alkaline, uncultivated soil with sparse vegetation, and 5 to 20 inches (13 to 51 cm) of annual rainfall. They postulate that the hot summer temperatures sterilize the upper 4 to 6 inches (10 to 15 cm) of soil, eliminating all competitive organisms, and leaving this layer a good medium for growth of C. immitis after the next rainfall; the arthrospores from this growth are then carried off by wind during the next dry spell.

For the purpose of the present epidemiological study, the Tucson area in southern Arizona, lying in the heart of the endemic area for coccidioidomycosis, was chosen as the exposure site. The infectivity for man in this area (Fig. 4) approaches 70% in long-time residents, and for cattle is closer to 80% (18). The facilities of the College of Agri-

Fig. 3. *Area of the United States covered by the Lower Sonoran Life Zone (17). (Courtesy of Keith T. Maddy.)*

culture, The University of Arizona, Tucson, were made available for the project, under direction and cooperation of the Department of Animal Pathology.

PHYSICAL SET-UP OF ANIMAL EXPOSURE SITES

Three fenced-in (chain-link) areas, approximately 30 by 40 ft in size and approximately 100 ft apart, were constructed in a shallow arc arrangement, affording each enclosure exposure to the prevailing wind (Fig. 5–7). The pens were located at the University's Casa Grande Farm in the Santa Cruz River basin (a venturi-like geographical site, the local hills of which funnel the prevailing wind through the area). This farm contains feed-lots in which practically all cattle imported from nonendemic areas eventually become infected with *C. immitis* (19).

In each of the three enclosures (Fig. 8), eight mixed-breed dogs were allowed free run of the area, and eight monkeys (*Macaca mulatta*) were confined in open cages (Fig. 9) approximately 26

inches (66 cm) above ground level, under appropriate shelter. All animals remained at the open exposure sites for a period exceeding 1 year, unless they became infected with *C. immitis*. As the animals became infected, they were immediately removed from the exposure site and were replaced with reserve, susceptible animals.

PROCEDURES FOR DETERMINATION OF NATURAL INFECTION

Two dogs and two monkeys from each exposure pen (one-fourth of the population of each species) were subjected to coccidioidin dermal sensitivity tests, agar-gel immunodiffusion precipitin tests (24), and thoracic radiographs each week. This provided a population observation turnover once every 4 weeks. Each time an infection was noted, tests were immediately repeated on all animals. In addition, all animals were critically observed, several times a day, for clinical signs of infection.
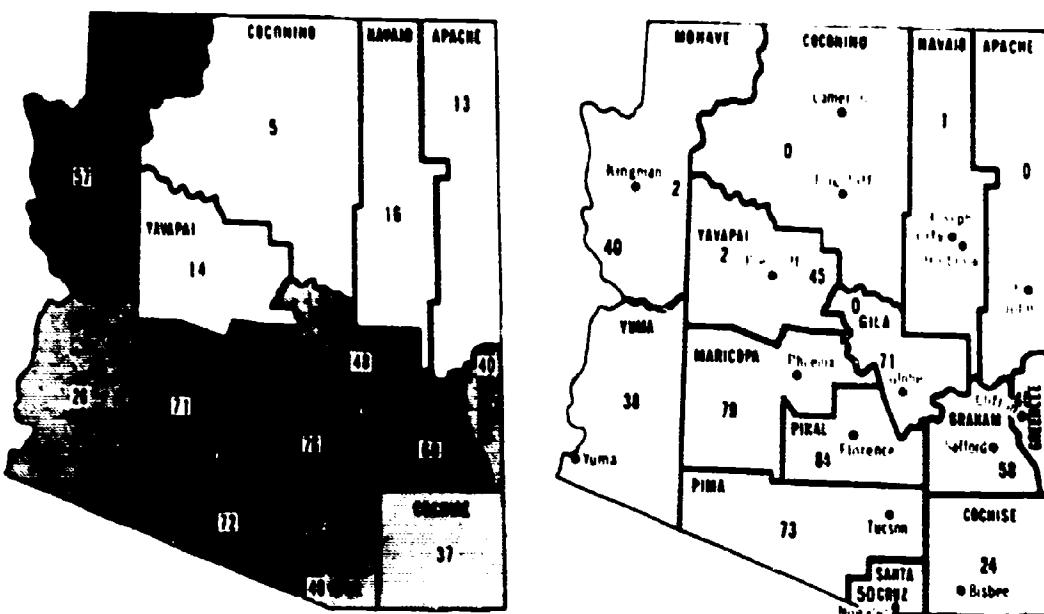
FIG. 4. *Maps of Arizona, showing infectivity (per cent) for man (left) and cattle (right, 18). (Courtesy of Keith T. Maddy.)*

CLINICAL AND LABORATORY OBSERVATIONS OF NATURALLY INFECTED ANIMALS

As each infection was noted, the animal in question was immediately removed from the exposure site and placed in air-conditioned quarters at another location (The University's Campbell Avenue Farm, noted for its low infectivity rate; R. E Reed, *personal communication*) to lessen the chance of further exposure to the organism. It remained under observation until the termination of the study. Changes in erythrocyte sedimentation rate, packed cell volume, per cent hemoglobin, total and differential leukocyte count, development of complement-fixation antibodies, and immunodiffusion precipitin titer were recorded weekly. Rectal temperatures were taken daily, and thoracic radiographs were made at 4-week intervals.

CONTROL ANIMALS

Controls for this study consisted of four types.

Ground Controls

Five monkeys were caged at ground level (physical contact with the soil) for 6 months (June to December 1964) of the 1-year period, at one of the exposure sites, to equate any differences in the monkey infection rate (housed several feet above ground level) and the dog infection rate (having free access to the soil).

Environmental Controls

Uninfected, susceptible monkeys and dogs were maintained at the Campbell Avenue Farm (where reserve susceptible animals were housed) and examined during and at termination of the study to assure that the naturally infected animals were not receiving any further exposure to *C. immitis* after removal from the exposure sites (Casa Grande Farm).

Experimentally Infected Controls

Ten monkeys and eight dogs were inoculated intratracheally with 10 or 100 *C. immitis* arthrospores from a culture isolated from the soil of the area under study. These animals received the same clinical and laboratory tests as the naturally infected animals. In addition, determinations were made each week of serum amylase, serum glutamic-oxaloacetic and serum glutamic-pyruvic transaminases, total serum protein, and serum protein fractions.

Animals from Former Experimental Studies

As a further comparison of naturally and experimentally infected animals, similar data from

Fig. 5. *Photograph of the Tucson area. Note dust cloud visible at the base of the mountain.*

former studies (2, 4) of monkeys exposed, via the respiratory route, to aerosols of *C. immitis* arthrospores (10 to 10,000 organisms) and dogs (R. E. Reed, *personal communication*) inoculated, via the intratracheal route, with 10 to 100,000 organisms were assembled for use at termination of the present study.

PATHOLOGICAL STUDIES

At termination of the study (52 to 54 weeks), complete necropsies were performed on all of the naturally exposed animals (both infected and non-infected) and all of the controls. Gross pathology was recorded and photographed; the lungs and any suspicious lesions were examined by impression smears for presence of *C. immitis* and were cultured on Mycobiotic agar (Fisher Scientific Co., Pittsburgh, Pa.) for recovery of the organism; tissues for histological study were fixed in 10% buffered formaldehyde, impregnated with paraffin, serially sectioned, and stained with the Giemsa and Gomori silver methenamine stains.

GEOPHYSICAL STUDIES

Climatic Factors

Throughout the 12-month period (October 1963 to October 1964), continuous wind speed and direction (anemograph) and relative humidity and temperature (hygrothermograph) recordings were made. Total precipitation was measured each day.

Soil and Aerobiological Studies

Soil in the area of the exposure site was analyzed to determine percentages of sand, silt, clay, and organic content, the hydrogen ion content, and both qualitative and quantitative analyses of salinity (Na, Ca, Mg, K, Cl, SO_4, CO_3, HCO_3, etc.). In addition, four to eight surface and subsurface soil samples were collected, bimonthly, in and around the exposure pens. These were plated directly on Mycobiotic agar, and aqueous suspension (1:10 dilutions) were injected intranasally and intraperitoneally into mice (six per sample) for recovery of *C. immitis*. Mycobiotic agar plates
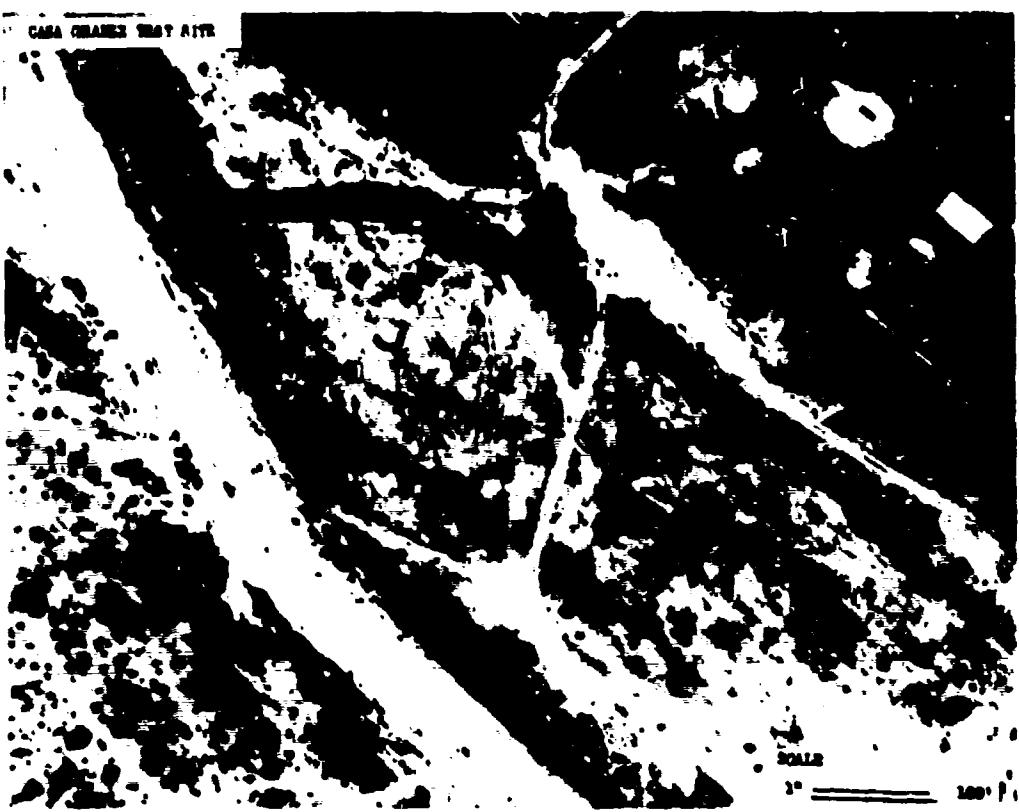
FIG. 6. *Aerial photograph of the Casa Grande exposure area. The three exposure sites (location of pens) are indicated by arrows. Note dry bed of the Santa Cruz River.*

were exposed to the atmosphere each day for recovery of the organism from the air.

COMPARISON OF METEOROLOGICAL DATA FROM THE PRESENT AND OTHER EPIDEMIOLOGICAL STUDIES

The meteorological factors (Table 1) were very similar to those of former studies in the same general region (South Central Arizona). Maddy (17) and Hugenholtz (12), after analyses of weather data covering periods of 10 to 20 years in several locations in South Central Arizona, reported mean July temperatures of 80 to 90 F (26.7 to 32.2 C), compared with 88 F for the present study. Their mean maximal and peak temperatures were recorded as 105 and 110 F, respectively, compared with 101 and 110 F in our study. Mean January temperatures of 50 to 55 F in the former studies were slightly higher than the 47 F mean temperature at Casa Grande Farm. The average yearly rainfall of 9 inches reported by Maddy, and 6 to 10 inches by Hugenholtz, was somewhat less than the 12.5 inches we recorded.

The only climatic factors that might have affected this study adversely were mean winter temperatures about 10 degrees below normal (from January on), with a very late, cold spring and an excessive amount of rainfall during August and September (total of 9 inches).

RECOVERY OF C. IMMITIS FROM AIR AND SOIL

Prevailing winds throughout the area were not very consistent during this study. Wind direction varied greatly, but analysis of the anemograph data indicated that most wind came from the west, through the north quadrant, rather than from the south through west as expected. Wind speeds sometimes as high as 34 mph were recorded. All attempts to isolate C. immitis from the air met with failure; however, two soil samples collected in August 1964 and two in October 1964 were positive for the fungus. The animal exposure area was well bracketed by these four positive soil samples (Fig. 10); one was actually collected from within exposure pen 3.

SEASONAL MORBIDITY OF MONKEYS AND DOGS

Five of 34 monkeys and 29 of 50 dogs became infected with *C. immitis* during the 12 months (Fig. 11). The majority of these infections (20 of the 29 infected dogs and all 5 of the infected monkeys) were diagnosed during the cooler months of November through March. Infections in an additional group of seven dogs and one monkey were

FIG. 7. *Ground view of the same area as in Fig. 6. Note Santa Cruz River meandering through center of photograph and exposure pens in upper left, near horizon.*

FIG. 8. *View of one of the three exposure pens. The solid-appearing structure extending up the fence from the ground is made of louvered aluminum, and permits the entry of wind and dust. The roofed structure visible inside the fence is the monkey shelter.*

FIG. 9. *Close-up view of the monkey shelter. The buttery consists of four cages (housing two monkeys each), completely open on four sides and partially open on a fifth side. Note one of the oil-drum dog shelters in the background. The dogs have free run of the fenced-in area (30 by 40 ft).*

TABLE 1. *Comparison of weather conditions in three epidemiological studies*

Observation	Maddy (17)	Hugen-holtz (12)	Present study
Mean July temperature	90	80-90	88
Mean maximum Temperature	105		101
Peak temperature		110	110
Mean January temperature	50	50-55	47
Minimum temperature			14
Rainfall (inches per year)	5-20 (Avg 9)	6-10	12.5

classed as equivocal. These eight animals may have received an exceedingly small exposure to the fungus. In these instances, however, either an equivocal dermal sensitivity was never corroborated by serological or histological reactions, or else the serological titers were very low and inconsistent. Seven of these eight questionable infections also occurred from November to March; the other, in one of the five monkeys housed at ground level and having access to the soil, was noted in October 1964.

PATHOGENESIS IN MONKEYS

Natural Infections

Only three of the five naturally infected monkeys developed precipitin titers, although all five eventually became complement fixation (CF)-positive (Table 2). Only two monkeys exhibited histological lung changes indicative of coccidioidomycosis; these were very minor. Lung cultures of all monkeys were negative for *C. immitis*. The five infected monkeys remained in apparent good health during the 12-month period, showing no clinical signs of disease; their serological titers (both precipitin and CF) were comparatively low (mean maximum of 1:8, with a range of negative to 1:64); very little evidence of infection was noted in X-ray studies.
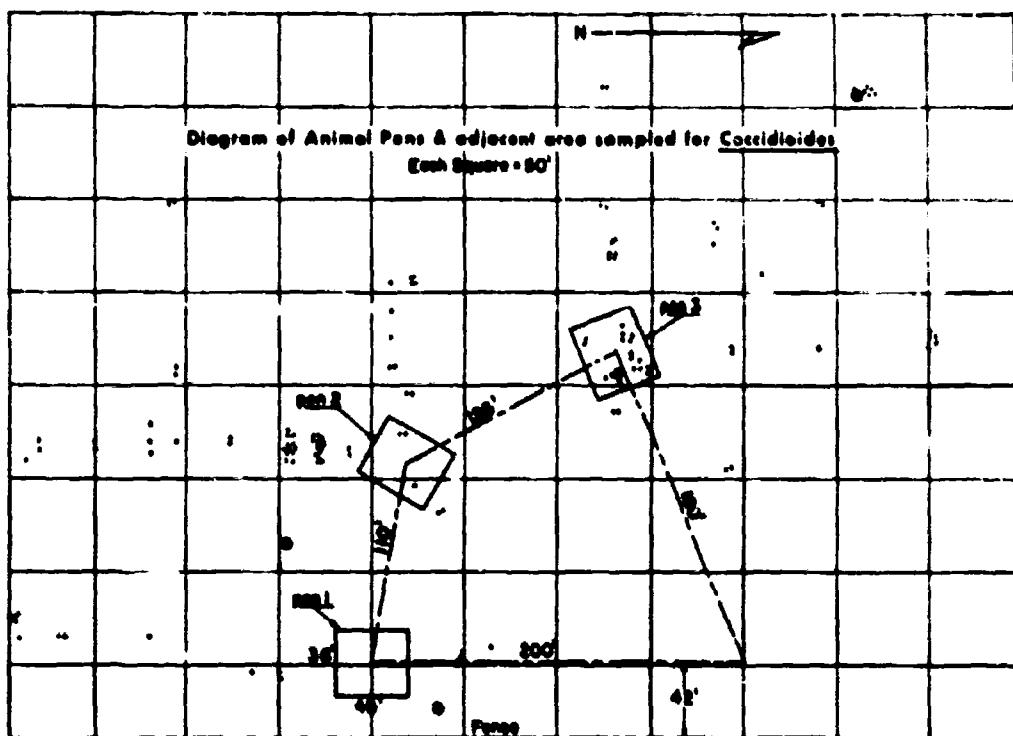
FIG. 10. *Pattern of soil samples collected in the exposure area. Dots indicate samples (the four positive samples are circled). Note bracketing of exposure pens by the positive samples (one collected from within pen no. 3).*

Comparative Pathogenesis of Naturally Infected and Intratracheally Inoculated Control Monkeys

Table 3 indicates that the 10 monkeys experimentally infected via the intratracheal route with 10 C. *immitis* arthrospores developed more serious infections than the five monkeys that became infected through natural exposure. All animals in the inoculated group developed precipitin titers, compared with only 60% of those infected naturally. Although the mean titer was the same (1:?) in both groups, no precipitin data were available for the three inoculated monkeys with the most extensive infections. Such data should have substantially increased the mean titer of the inoculated group.

Of the inoculated group, 40% were clinically ill, as indicated by weight loss, listlessness, and coughing. This included one animal that died from the infection 24 days after inoculation and two animals that, upon autopsy, were judged unable to have survived. All animals in the naturally exposed group were considered to have subclinical infections.

Upon autopsy, 80% of the inoculated monkeys exhibited histological changes in the lung indicative of coccidioidomycosis, including presence of the organism, compared with only 40% of those developing natural infections. The fungus was recovered from 60% of the lung cultures from animals in the former group, but all cultures from the latter group were negative. Figures 12 and 13 graphically illustrate the more extensive lung involvement in the inoculated group.

Comparative Pathogenesis of Naturally Exposed Monkeys and of Monkeys from Former Experiments Receiving Respiratory Challenges by Inhalation of C. Immitis Aerosols

Again, as indicated by data in Table 4, less severe disease was noted in monkeys developing natural infections than in those infected experimentally. Monkeys in several former studies, receiving calculated inhaled doses of from 10 to 300 arthrospores, developed mean serological titers ranging from 1:128 to 1:512, respectively, compared with a mean of 1:8 exhibited by the
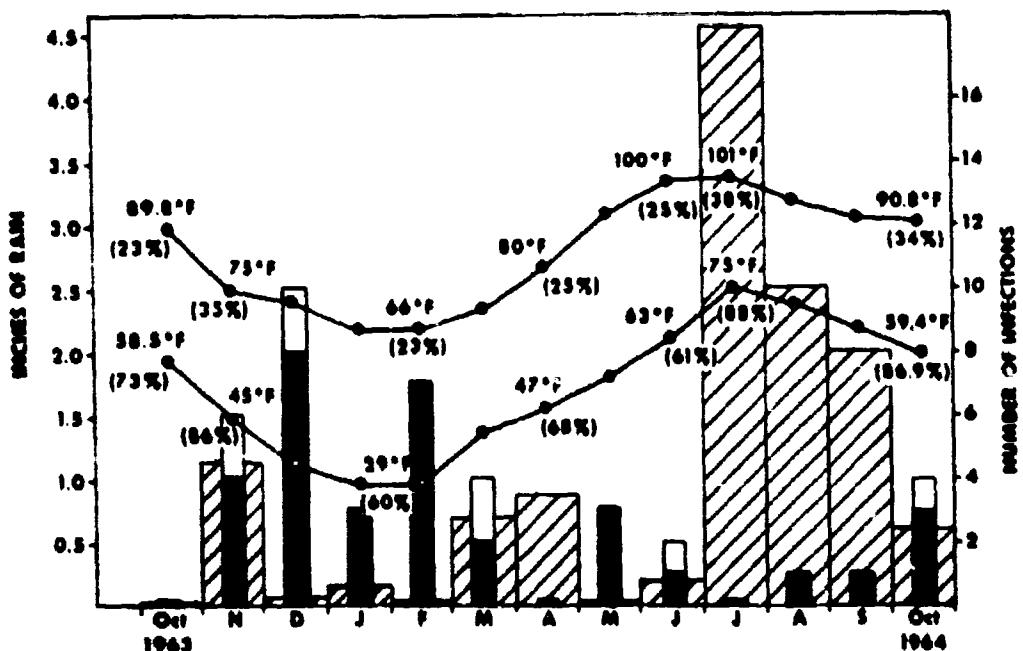
Fig. 11. *Seasonal morbidity of experimental animals. Cross-hatched bar indicates rainfall in inches; solid bar, number of infections; and solid lines, mean maximal and minimal temperatures for each month. The figures in parentheses represent per cent relative humidity at the temperature indicated. The open areas shown at the top of several of the solid bars indicate suspected infections that were never subsequently proved.*

TABLE 2. *Natural infections in monkeys and dogs*

Animal	No. exposed	No. infected	No. with positive skin test	No. with positive serology	No. showing histopathological changes	No. with positive lung cultures
Monkey	34	5	5	3	2*	0
Dog	50	29	20	22	18	3

* Very minor.

naturally exposed animals in this study. Moreover, a mortality of 30 to 40% in the former studies contrasted to the lack of mortality in naturally exposed animals in the present study.

PATHOGENESIS IN DOGS

Natural Infections

The extent of the disease, as well as the infection rate, was much greater in the naturally exposed dogs than in the naturally exposed monkeys (Table 2). Of the 29 naturally infected dogs, 20 developed dermal hypersensitivity to coccidioidin, and 22 exhibited positive serological titers (21 of these 22 were positive for both the precipitin and

CF tests). Upon autopsy, 18 showed histological lung changes due to *C. immitis*. In three instances, *C. immitis* was isolated from the lungs by culture. In 4 of the 29 infections, the disease was diagnosed by histological methods alone, since all clinical and laboratory tests on these four animals were negative.

The majority of the 29 infected dogs remained healthy in appearance throughout the observation period. Of the 29, 7 exhibited clinical signs of illness (weight loss, listlessness, and coughing), but these appeared to have recovered before the end of the experiment. From this standpoint, the remaining 22 infected dogs could be classed as subclinical cases of the disease.

Comparative Pathogenesis of Naturally Infected and Intratracheally Inoculated Control Dogs

In contrast to the findings for monkeys, the disease was more extensive in dogs infected by natural exposure than in those inoculated intratracheally (Table 5). None of the dogs in the inoculated groups (10 or 100 arthrospores) developed precipitin titers, compared with 75% of those infected naturally (mean titer of 1:16, with a range of negative to 1:512). The majority of the

TABLE 3 Comparison of naturally and experimentally infected monkeys

Exposure	Positive skin test	Positive serology		Clinical signs of illness	Lung culture positive	Histologically positive
		PPN	CF			
10 arthrospores (intratracheal inoculation)	100%	100%; mean titer,* 1:8 (±1:2 to +1:32)	100%, 1:256 I* 1:2 1:256 I 1:2 1:4 I 1:2 1:4 I 1:2 1:2 I 1:2	40%; one died at 24 days; two judged unable to survive	40%	80%
Natural exposure	100% (one equivocal)	60%; mean titer, 1:8 (negative to 1:64)	100%; 1:32 1:8 1:4 1:4 I 1:2	None	None	40%

* No data for the three animals with the most extensive infections, which should have increased the mean titer of this group
† I = incomplete reaction.

FIG. 12 Comparative X-rays of naturally infected (top) and experimentally infected (bottom) monkeys. Note areas of pulmonary infiltration in the majority of animals receiving intratracheal inoculation of 10 arthrospores, as compared with the relatively clear lungs of the naturally infected monkeys.
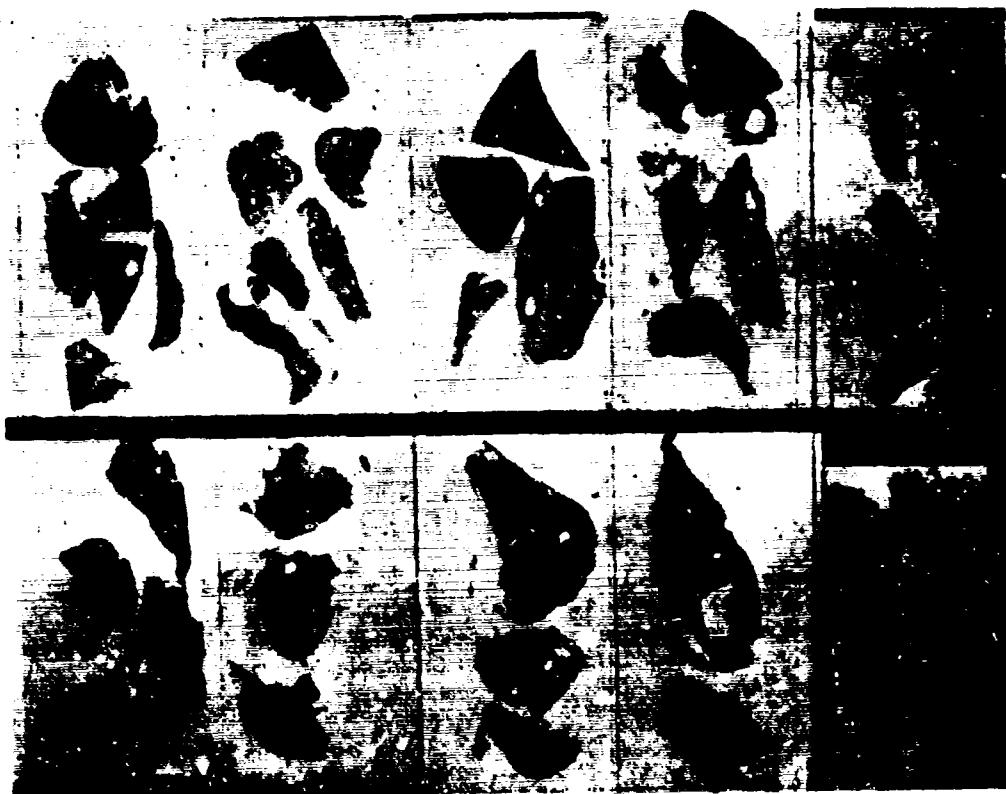
FIG. 13. *Comparative histological sections of naturally infected (top) and experimentally infected (bottom) monkeys. Note particularly the lung tissue (comparatively normal appear ng in the naturally infected monkeys, as compared with the large areas of consolidated lesions in animals inoculate.: intratracheally with 10 arthrospores).*

animals in all three groups eventually developed CF titers; however, titers for the inoculated groups were negligible (possibly equivocal) compared with a mean titer of 1:8 and a range of negative to 1:256 in the naturally infected group. Clinical signs of illness were evident only in the naturally i...cted group (approximately 25%), but these were relatively mild.

Upon autopsy at termination of the observation period, *C. immitis* was not isolated by culture from the lung of any of the inoculated dogs, but was found in the lungs of 3 of the 29 dogs infected by natural exposure. Of the inoculated dogs, 38% exhibit d histological changes in the lung indicative of coccidioidomycosis, compared with 62% of those exposed naturally. Although not too much difference was noted on X-ray examination of inoculu d and naturally exposed dogs (mainly pulmonary lymphadenopathy in both groups), Fig. 14 illustrates graphically the difference in histological changes in the lungs of the two groups. Although the disease in naturally infected

dogs was more extensive than that in naturally infected monkeys, the 29 dog infections were con sidered to be relatively mild illnesses.

Comparative Pathogenesis of Naturally Exposed Dogs and of Dogs in Former Studies Receiving Intratracheal Inoculations of C. immitis

In former studies (R. E. Reed, *personal communication*) of pathogenesis of coccidioidomycosis in dogs, approximately 100 animals of various age groups (from 6 weeks to 6 months) were given intratracheal inoculations of graded doses (from 10 to 100,000) of *C. immitis* arthrospores. Unfortunately, no serological data are available on these dogs, but it may be seen in Table 4 that inoculation of 10 or 100 arthrospores resulted in 10 or 20% mortality, respectively, as compared with no mortality in the 29 naturally infected dogs.

ENVIRONMENTAL CONTROLS

There was no clinical, serological, radiological, histological, or cultural evidence of coccidioido-

TABLE 4. *Comparison of naturally exposed infected animals with experimentally infected animals*

	Monkey			Dog		
Dose[a]	Mean[b] maximal titer	Mortality	Dose[c]	Mean maximal titer	Mortality
		%			%
Natural exposure (unknown)	1:8 (negative to 64)	0	Natural exposure (unknown)	1:16 (negative to 512)	0
10	1:128 (64:256)	40	10	—[d]	10
50-100	±1:256 (128:256)	30	100	—	20
300	1:512 (128:1,024)	40	1,000	—	47

[a] Numbers refer to aerosol arthrospore dose (experimental; references 4, 14). These data represent a total of 50 monkeys, at 5 to 10 monkeys per dose group.
[b] Immunodiffusion precipitin test. Numbers in parentheses indicate spread.
[c] Numbers refer to intratracheal arthrospore dose (R. E. Reed, *personal communication*).
[d] Test not made.

TABLE 5. *Comparison of naturally and experimentally infected dogs*

Exposure	Positive skin test	Positive serology[b]		Clinical signs of illness[c]	Lung culture positive	Histologically positive
		Precipitation	CF			
	%			%	%	%
100 arthrospores (it)[a]	100	None	100% 2 + 1:4 ±1:2 ±1:2 ±1:2	None	None	50
10 arthrospores (it)	100	None	75% +1:4 ±1:4 ±1:2 Negative	None	None	25
Natural exposure	69	75%; mean titer, 1:16 (negative to 1:512)	74%; mean titer, 1:8 (negative to 1:256)	24	10	62

[a] Intratracheal inoculation.
[b] Figures in parentheses indicate span.
[c] Weight loss, listlessness, cough.

mycosis in any of the monkeys or dogs maintained at the Campbell Avenue holding area as environmental control animals.

DISCUSSION AND CONCLUSIONS

Previous epidemiological studies (12, 17, 31) have covered longer periods of time (10 to 20 years) and have included many more infections than the present study. These factors would tend to smooth out any inconsistencies, such as those possibly caused by freak weather conditions affecting growth of the fungus in the soil, or its dispersal by the wind, during any one year. Most observers have reported two peaks in the human infectivity rate for coccidioidomycosis: one during the hot, dry summer months, and another occurring in late fall to early winter. The lack of a summer infectivity peak in this study may have been due to the unusual amount of rainfall during July, August, and September 1964 (75% of the total for the year: Fig. 11). This period was noted for the frequency of rains, lack of drying between precipitation, wet, packed soil, and lush growth of weeds, all of which would discourage dissemination of the fungus by the wind.

The peaks in the infectivity rates for both species of animals in the present study were consistent in that all of the monkey infections, and
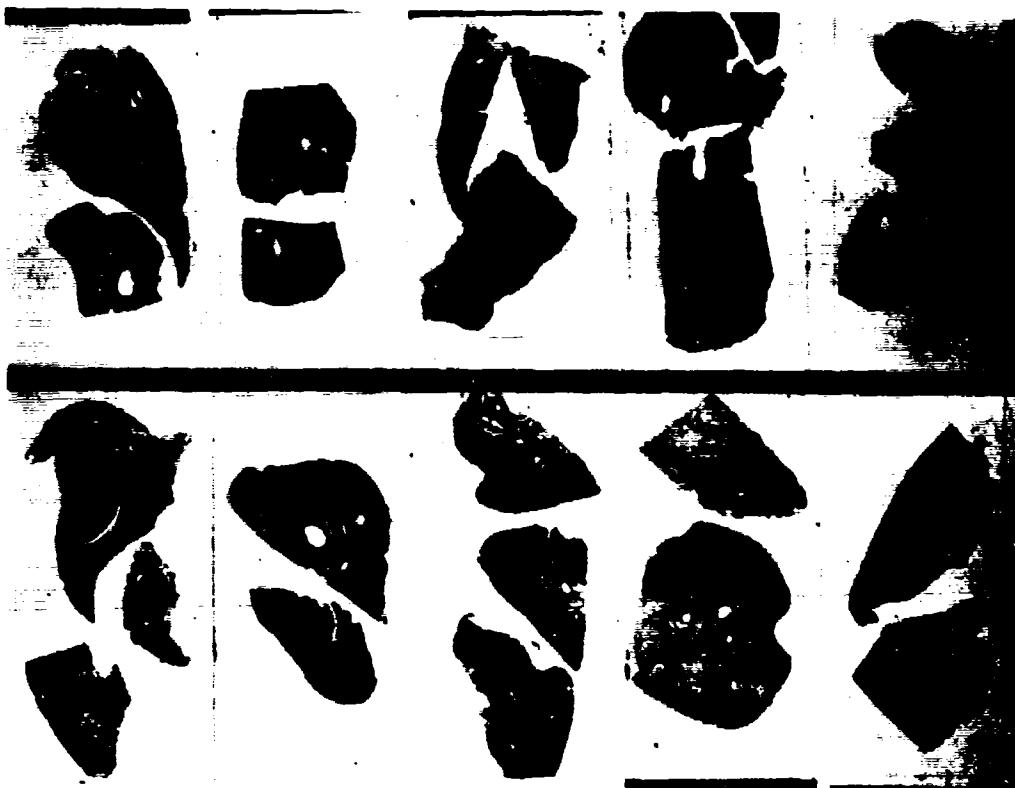
FIG. 14. *Comparative histological lung sections of naturally infected (top) and experimentally infected (bottom) dogs. The first two sections on the left (bottom row) received 100 arthrospore intratracheal inoculations; the next two, 10 arthrospores; and the section at the extreme bottom, right, was from an uninoculated control dog. Note visible lung lesions in all naturally infected animals, and in only one of the four inoculated dogs shown here.*

20 of the 29 dog infections, were diagnosed from November to March. Although the infectivity rate was much higher for dogs than for monkeys, this undoubtedly was the result of their free run of the exposure pens (direct contact with the soil), their habit of fighting among themselves, and their constant digging (sometimes burrowing as much as 1 ft in the ground); all of these tended to stir up the dust and to uncover the fungus during the seasons when it would be expected to be 6 to 12 inches below the surface of the ground (8). Infection of dogs from contact with the soil was further indicated by the infectivity pattern; 19 of the 29 dog infections occurred in pen 3, the remaining 10 were divided between pens 1 and 2. Not only has it been pointed out before that growth of *C. immitis* in the soil is spotty rather than universal, even in heavily endemic areas (8), but also one of the few positive soil samples (Fig. 10) in this study was collected from within pen 3.

For the purpose of this study, the monkey was considered a more valid indicator ("biological air sampler") of the human infectious dose than the dog, because, being housed several feet above ground level, the monkeys undoubtedly became infected by normal airborne arthrospores rather than from close contact of the nose with the soil. However, an analogy in man to dog infectivity higher than that of the monkey might help to explain the more severe disease found in ground-construction workers and people engaged in agricultural pursuits (10), who also turn over the soil and stir up large amounts of dust in their daily occupations. Possibly man also contributes to the peak infectivity period shown in human infections during the summer months by his outdoor recreational activities, such as hiking, picnicking, digging for fossils, etc. (27).

In analyzing the present study, we must not lose sight of the fact that virulence differs among various strains of *C. immitis* (11, 11a), and also that the natural infections in the monkeys and dogs may have resulted from repeated exposure to extremely small doses (one to two arthro-

spores) before being detected by dermal sensitivity or serology, thereby building up a certain amount of immunity. However, a presumption that the infections originated from *large doses* of arthrospores of *low virulence*, would not be in accord with the *extreme* difficulty in isolating the organism from the air, since low virulence and low viability are not synonymous, and the presence of large numbers of arthrospores in the atmosphere should have been indicated by air sampling.

The authors realize, fully, the limitations of an experimental epidemiological study of this type, but considering the marked differences in histopathology, the lack of mortality in either species, and the fact that clinical signs of illness were lacking in the monkey (an extremely susceptible animal) and were relatively mild in the few dogs that exhibited them, we feel that the naturally acquired disease in these animals resulted from very small infectious doses; 10 or fewer arthrospores in the monkeys, and from 10 to more than 100 arthrospores in the dogs, although the individual dose range received was probably much greater in the dogs than in the monkeys.

The pattern of infections in the monkeys, dogs, and in man might well be compared: (i) there are large percentages of subclinical or asymptomatic infections in all three species (70% of the infected dogs, 100% of the infected monkeys, and 60% of infected human subjects); and (ii) the more severely affected dogs are analogous to the more severely affected construction and agricultural workers, since they all actively stir up the dust during their daily existence, whereas the less severely affected monkeys might be compared with the average human infections.

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Discussion

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The preceding paper reveals the difficulties of field experiments in contrast to experiments conducted in the laboratory. The authors have constructed an experimental exposure chamber in the open to look for natural infections in a highly endemic area in Arizona. Their experimental design was quite satisfactory, although one could suggest they might possibly have used cynomolgous monkeys rather than mulatta. Their previous experiences with the mulatta, however, does indicate its sensitivity to infection.

Having set up this natural infection experiment, the authors awaited the infections. It turned out that 15% of the monkeys and 58% of the dogs

were infected during the course of 1 year. Unfortunately, the year encompassed a year of exceptionally heavy rainfall, during which 12.5 inches fell, an amount much higher than that customary in the area (6 to 10 inches). They found the expected number of infections in the early part of the year, but there were very few infections in the latter part of the year, during which the heavy rainfall occurred. If the observations had continued for another year, it is quite probable that after the period of heavy rainfall they would have encountered a period of heavy infection.

On the whole, one is inclined to think that the

authors' data are perhaps more satisfactory than they claim. Since, for the area studied, Hugenholtz had reported (2) a yearly infection rate of 5.4% and an illness rate of 2 to 3 per 1,000 population, one should not be at all discouraged by finding that almost 15% of the monkeys were infected and more than half of the dogs. The results, therefore, would indicate that the area in which they were working was exceptionally heavily infected and that the results were quite in line with those expected from the conditions of the experiment.

The observations on the serological and clinical response of the infected animals do suggest low dosage infection. None of the 5 monkeys and only 3 of the 29 dogs showed positive cultures at autopsy.

Perhaps it would be well to consider separately the results of the exposure in monkeys and in dogs. The clinical picture found in the monkeys which became infected after natural exposure was less severe than that of animals infected artificially with 10 organisms. On the other hand, experimentally infected animals showed a higher mortality and higher antibody titers.

With dogs, the frequency of clinical symptoms was higher, as were positive cultural and histologic findings, among the naturally infected animals than among those artificially infected. This is probably to be expected, inasmuch as the dogs were allowed to roam free and dig in the soil, which would probably mean they would secure heavier exposure.

It is interesting to note that, although no illness occurred among the infected monkeys, 35% of the infected dogs had illness; this agrees very well with the observations made by Smith et al. (3) among naturally infected man.

When one considers the wards full of acute coccidioidomycosis in the air bases at Arizona, one would expect that large numbers of naturally exposed animals would develop infection and illness. One is, therefore, somewhat disappointed until one considers that the large numbers of cases reported by Hugenholtz (2) really represent a low infection rate per 1,000 persons stationed at the base, since fewer than 3 per 1,000 population were admitted to the hospital during any one month.

When one considers again the clinical implications of this work, one is struck by the lack of severity of disease in the monkeys and dogs versus man. This must be related to dosage, since there

is evidence that both dogs and monkeys do develop severe disease after experimental infection. If one recalls the high frequency of severe clinical disease reported in the epidemics of coccidioidomycosis where exposure was known to be heavy, one is forced to conclude that clinical severity reflects in part at least heavy dosage of fungi. This is supported by the fact that the dogs, which had closer contact with the soil, had higher infection rates and more severe disease than the monkeys whose cages were elevated off the ground. A corrolary of this observation would appear to be that the exposures in the Army personnel who became ill must of necessity have been heavy. Similar relationship of degree of exposure to degree of illness has been reported in histoplasmosis epidemics.

Still another factor is the difference in clinical symptoms related to the age of the individual at the time of infection. This is still largely an unknown factor and was not explored in these trials.

Finally, one comes to the contribution of these studies to the basic epidemiological question as to where one does get infected with *Coccidioides*. Is it by walking down the streets of Phoenix or Tucson or Bakersfield and inhaling the air, or is it necessary to go to a point source where the organisms are growing and stir up an aerosol there? As most of you know, the evidence in histoplasmosis points to point source visits as the mechanism of infection. The studies reported here suggest that this mechanism is also operative in coccidioidomycosis and that the animals were infected from the local focus. This is supported by the soil isolations which were made in the cage area. These data, therefore, support the "local focus" theory of infection rather than the "generalized windblown" theory.

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Concluding Remarks

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Modern reviews on microbial aerosols and airborne infection were initiated by the publication of the monograph, "Aerobiology," in 1942, by the American Association for the Advancement of Science (1). In the following decade, William F. Wells summarized his life-long investigations of these topics in an important volume (6). Some 6 years later, his pupil, Richard Riley, and Francis O'Grady reconsidered the problems of the transmission of respiratory infection (5).

These publications, and the numerous studies during that same period of the pulmonary deposition and retention of inhaled aerosols (2), provided a much firmer foundation than existed previously for the experimental investigation of airborne infection and the interpretation of the resultant observations. In addition, there have been extensive advances during the past two decades in the apparatus and techniques for quantitative experimentation in these fields.

A consequence of these improvements has been the emergence of a wealth of novel information on many aspects of aerobiology, and the need for periodic reassessments of our progress, both experimental and interpretive. To meet this need, a series of conferences has been organized—the first held at Miami Beach in 1960 under the sponsorship of the National Academy of Sciences (3), the second held in Berkeley in 1963 under the sponsorship of the Office of Naval Research and the Naval Biological Laboratory (4), and the present meeting being sponsored by the Illinois Institute of Technology Research Institute and the U.S. Army Biological Laboratories.

The presentations at this year's conference already have been thoroughly discussed by experts. It is not our intention, in these concluding remarks, to review again, in detail, the findings reported by each of the speakers. Instead, we will try to focus attention on some of the broad subdivisions of our overall theme.

The proceedings of previous conferences in this field, as well as several individual scientific publications, have provided information on equipment and techniques for the generation, containment, and quantitative characterization of microbial aerosols. These are at present sufficiently reliable that aerosols can be formed reproducibly with a content of infectious organisms

as dilute as 1 to 10 cells per liter. Thus, with one exception, experimental equipment, per se, has not been a topic in this year's conference program. The exception is the subject of sampling. A notable deficiency in the past has been the absence of dependable, high-volume samplers for determining the organisms suspended in the air of hospital rooms, barracks, school rooms, etc. In these environments, transmission of infection is frequently observed, but the role of the airborne route is still questioned. The overall problem of the epidemiology of airborne staphylococcal infection in hospitals has been thoughtfully reviewed by Williams. May, Perkins, and Gerone, in their individual papers, have provided evidence that improved high-volume air samplers are practical. Future improvements in design and aditional experience in the use of such samplers will make substantial contributions to forthcoming studies of the transmission of infection in environments where the aerial content of organisms is quite low.

The impact of physical and chemical stresses on microbial aerosols has been discussed separately by Zentner and by Hatch. It is amply apparent from these reports, as well as several on the same topic in earlier conferences, that microbial aerosols are quite sensitive to changes in their water content, i.e., dehydration or rehydration, and alterations in the gaseous composition and temperature of their environment. Despite the progress made in these studies, it is not presently possible to formulate a theory which will permit the prediction and understanding of the changes in viability and infectivity such aerosols undergo when subjected to alterations in their environment.

The physical and physiological features of the respiratory tract have been reviewed by Proctor, Kass, Dalhamn, and Rylander. Proctor's development of novel techniques for measuring the air flow in the nasal passages gives promise of providing information of considerable value in studies of air pollution, as well as of the airborne transmission of infection. The clearance mechanisms of the respiratory system are often circumvented in experimental studies where highly infectious organisms are administered to the host, in dilute form and as micron-sized particles. Under natural circumstances, the role of the respiratory

tract as a portal of entry for airborne infectious organisms is probably influenced to a significant extent by these mechanisms.

The resistance of the host to infection may be significantly altered by metabolic disorders, debilitation, extensive surgery, immunosuppressive or radiation therapy, etc. This altered resistance may play an important role in the epidemiology of hospital-acquired infections, which Williams has discussed. The so-called normal individual may also be subjected to environmental stresses, such as the inhalation of air pollutants. Ehrlich has demonstrated an alteration in susceptibility to airborne infection of the mouse, when exposed to small amounts of nitrogen dioxide. It is too soon to comment on the relationship of these experimental observations to public health problems of man.

Saslaw, Hearn, and Miller, in individual presentations, have provided considerable additional evidence for the susceptibility of laboratory animals to infection by inhalation of aerosols of viruses or rickettsiae that, in nature, are transmitted by insect vectors. Saslaw has made a particularly thorough study of Rocky Mountain spotted fever in the monkey and has demonstrated the similarities in the disease induced through the respiratory portal of entry to the naturally occurring disease.

Aerogenic immunization is a commonly used technique in poultry husbandry, but has not been sufficiently well evaluated, thus far, to receive general acceptance in human medicine. Two studies have been reported at this conference, that of Yamashiroya using tetanus toxoid and that of Hornick using a live tularemia vaccine. The latter investigation is particularly noteworthy, since it has been possible to use both experimental animals and human volunteers as subjects, and to evaluate immunization by direct challenge with fully virulent organisms. Further work will be required to determine the relative merit of the aerogenic as compared with more conventional modes of vaccine administration. In addition, careful consideration should be given to defining the medical and physical conditions which would contraindicate use of the aerogenic technique.

There are a number of infectious diseases whose airborne characteristics can be studied only in laboratory animals. Investigations in man are precluded, since adequate means of control of the disease are not available. The present conference has heard two such investigations—that of Brachman on industrial inhalation anthrax and that of Converse on coccidioidomycosis. Both field investigations were preceded by thorough

laboratory studies of the airborne disease in the same species of animal that was later used in the field. As a result, an extensive body of knowledge was available from laboratory investigations on dose-response relationships, incubation period, histopathologica' and immunological alterations, etc., which permitted a comprehensive correlation and interpretation of the field results.

Tularemia has been a most useful model of a bacterial disease that may be investigated in man. Studies reported in earlier publications, as well as the present conference, have presented a wealth of information on the susceptibility of man to experimental airborne infection, the use of attenuated strains of *Francisella tularensis* as a live vaccine, and the efficacy of vaccines, prepared in a variety of ways, in protecting the host against respiratory tularemia. Sawyer has extended our knowledge of this disease by investigating the conditions under which a bacteriostatic antibiotic (tetracycline) is effective in its management. A regimen has been found which may be employed for prophylactic or therapeutic purposes.

One of the direct benefits of the 1960 Miami Beach Conference was the subsequent initiation of a cooperative program of research on human viral respiratory diseases by the National Institute of Allergy and Infectious Diseases and the U.S. Army Biological Laboratories. This collaboration has provided an opportunity to apply quantitative and reproducible techniques for the generation, sampling, and characterization of viral aerosols to the exposure of human volunteers, and the study of their laboratory and clinical evidences of infection and illness.

Gerone has commented on some properties of laboratory-generated viral aerosols used for inoculation purposes and has presented early results in evaluating the output of viral aerosols into the environment of an infected human host.

Couch summarized the dose-response relationships observed in exposure of human volunteers to aerosols of coxsackievirus A-21, rhinovirus NIH 1734, and adenovirus type 4, and has compared the results obtained with this mode of inoculation with those obtained after intranasal instillation of virus. He has provided evidence for the production of airborne virus by coughs and sneezes of the infected volunteers - probably sufficient contamination of the environment to transmit infection to other susceptible individuals. In an interesting preview of a field study still in progress, Couch has provided preliminary information on the transmission of coxsackievirus A-21 infection among men in a controlled barracks environment, with evidence that the airborne

route played a significant role in the high level of transmission observed.

These thorough and extensive laboratory and clinical studies, in which the response of man is quantitatively related to the dose of virus inhaled, have provided a firm base for the design and interpretation of the experimental epidemiological studies, in which the mode of transmission of the virus under natural circumstances is the main focus of the investigation.

It is amply clear that this conference has served a valuable function in providing a critically prepared summary of progress in our knowledge of microbial aerosols and airborne infection. At the same time, the conference and the discussion it has stimulated will unquestionably influence the course of future investigations in these fields. The plan for prompt publication of the Proceedings in *Bacteriological Reviews* is an important mechanism for assuring the attainment of this objective. We congratulate all who have contributed to the success of this conference.

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